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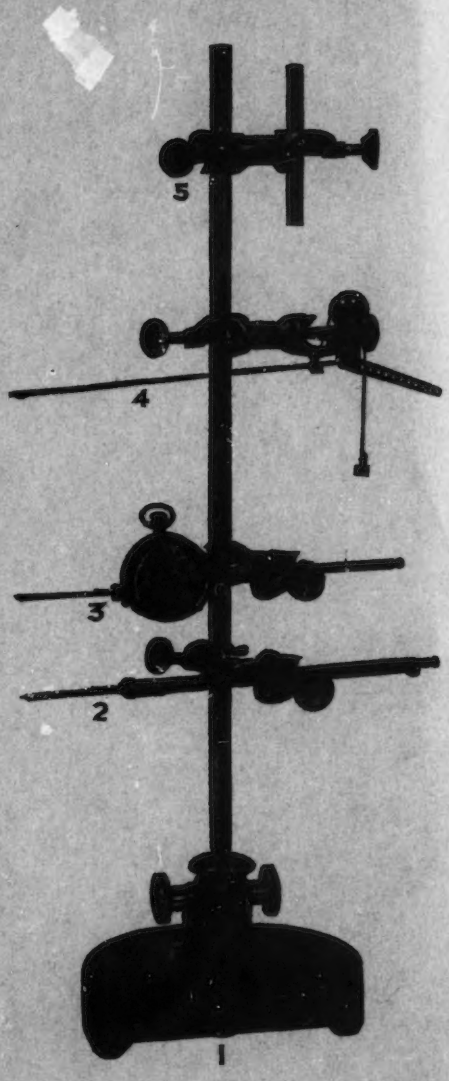

JOSEPH BECKER

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REGULATION OF RESPIRATION

XX. A Quinhydrone Electrode Vessel and Adapter and Blood and Tissue Fluid Collection Tubes Designed for Ch Determinations on Small Samples Without Loss of Carbon Dioxide[1]

ROBERT GESELL

From the Department of Physiology, University of Michigan, Ann Arbor

Received for publication June 23, 1928

The rapidity and convenience of the quinhydrone electrode as developed by Biilmann and his associates (Biilmann, 1923; Lester, 1924; Biilmann, 1925; Biilmann, Jenesen and Pedersen, 1925; Biilmann and Jenesen, 1927; Biilmann and Katagiri, 1927) for the measurement of hydrogen ion concentration recommends its use in many biological problems. Recently the application of the method has been extended to pH determinations of blood serum (Cullen and Biilmann, 1925) and the validity of the findings and the necessary correction for the effect of blood protein have been established (Cullen and Earle, Earle and Cullen, 1928).

In the use of this electrode which has been exceedingly helpful in our problems on blood and cerebro-spinal fluid acidity (Gesell and Hertzman) erratic E.M.F. readings have been encountered at times sufficient to discredit the findings of a single experiment. On the other hand such experiences were more frequently contrasted with a set of readings throughout a long series of samples of approximately the accuracy described by Cullen and Earle. We have been inclined to attribute the erratic E.M.F. readings to variability in behavior of the electrode. In the employment of small samples another difficulty is encountered—that of preserving and transferring the sample to the electrode without loss of carbon dioxide. This holds particularly for poorly buffered fluids such as cerebro-spinal fluid. Small samples (0.20 cc.) kept under oil in diminutive open tubes gave progressive alkaline readings with successive determinations from the same

[1] Demonstrated before the American Federation of Societies for Experimental Biology, April 13, 1928, Ann Arbor, Michigan.

sample. The methods devised to overcome these difficulties are here described. It should be mentioned, however, that the quinhydrone electrode has not been checked against the hydrogen electrode. The findings of Cullen and Biilmann, Cullen and Earle on the validity of the absolute values yielded by the electrode have been referred to above.

Two possible sources of error in the use of the quinhydrone electrode are variations in E.M.F. due to diffusion of potassium chloride from the potassium chloride bridge in the capillary up to the electrode and diffusion of carbon dioxide from the sample into the overlying air space above the electrode and capillary. By providing constant mechanical conditions with

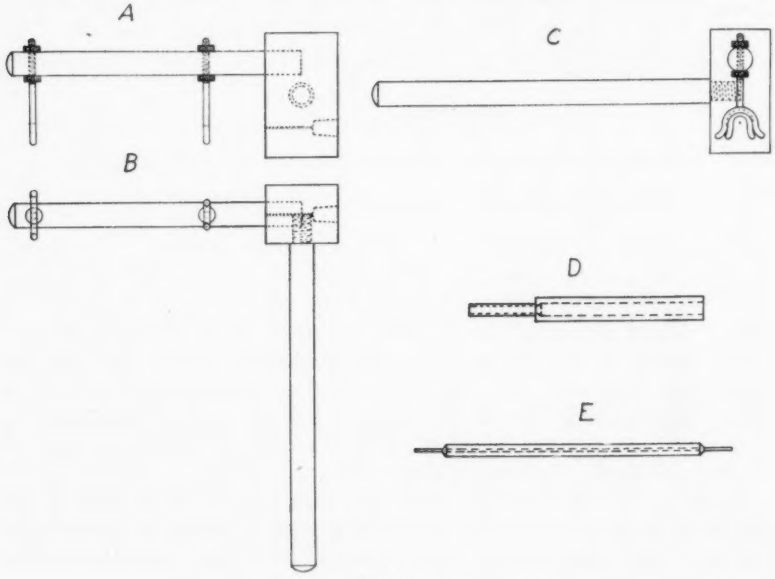

Fig. 1

each determination these possible sources of error should at least be avoided and other unforeseen advantages gained.

To obtain these advantages the capillary electrode was made of transparent bakelite accurately turned on the lathe (see fig. 1). The accompanying figure drawn to scale shows the construction in detail. (The total length of D is 62 mm.) A piece of no. 24 B and S gauge platinum wire or 24 karat gold wire extending through the centre of the bakelite plunger serves as the electrode and also provides electrical contact with the potentiometer without use of mercury. The dangers of mercury are thus avoided. The lower free end of platinum is carefully polished with a fine

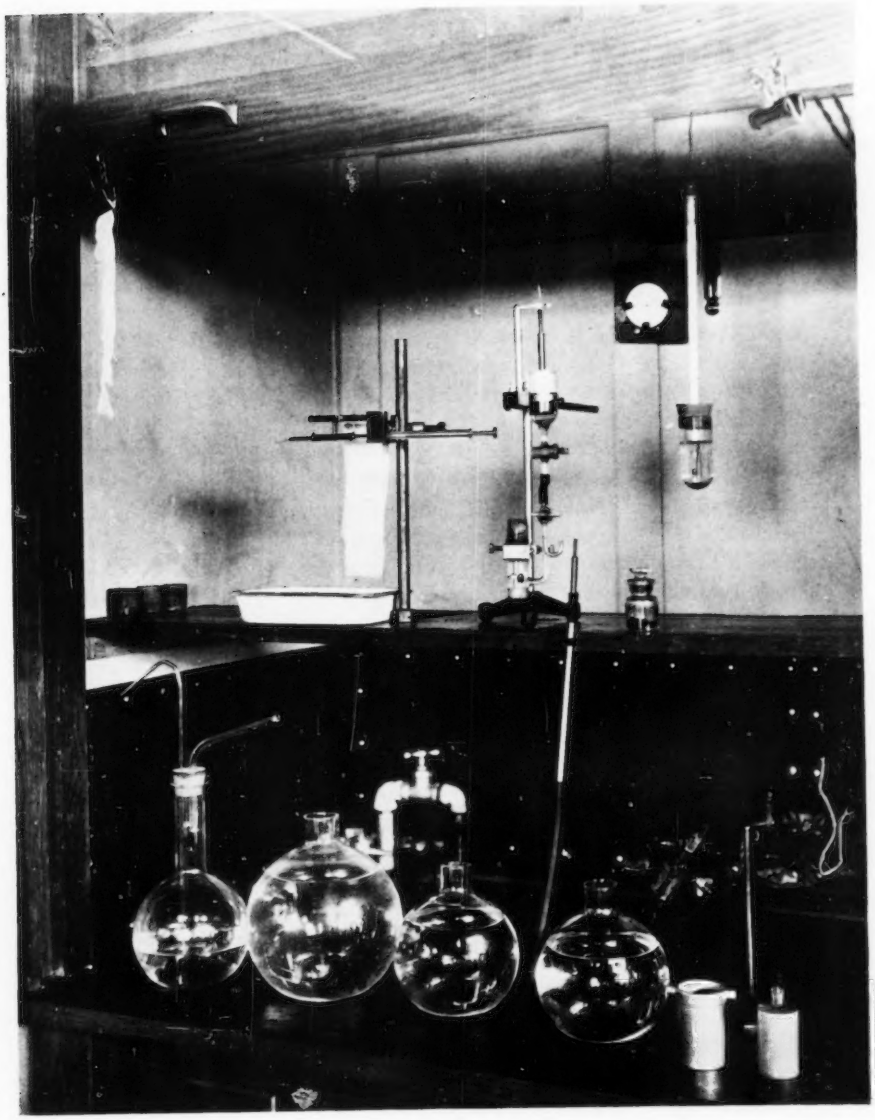

Fig. 2

Arkansas stone and the point well rounded. It is then sealed into the plunger and covered at the base for about 3 mm. with a thin smooth layer of De Kotynsky cement. It is advantageous to obtain a smooth covering of De Kotynsky as it permits a more uniform coating of quinhydrone before each determination. This is easily accomplished with a cautery knife held some distance from the electrode after the cement has been applied. If the electrode is held vertically the excess cement tends to run to the bakelite. The other end of the plunger is sealed tightly to the plunger. The barrel accommodates the plunger not too snugly and the capillary takes the electrode freely so that a small but ample amount of sample may be forced around the platinum wire. The plunger is always pushed to the bottom of the barrel which completely eliminates the air space above the sample. The loss of carbon dioxide from above is thereby virtually excluded. The electrode must always of necessity reach the same level in the capillary sample and since the electrode is carefully centered in the capillary, physical conditions about the electrode should be highly constant. The sample is forced up the capillary about the electrode until it appears at the pinhole escape which is 5 mm. above the lower end of the barrel. Capillary attraction in the space between the barrel and plunger supports the sample in the capillary. The electrode vessel is transferred to the KCl bridge where the lower end of the capillary is allowed to rest on a block of bakelite in the KCl. The block is adjusted so that the lower end of the barrel is just immersed, which leaves the pin hole escape above the KCl. Salt error from diffusion is thus avoided. The arrangement for holding the electrode in position at the KCl bridge is shown in figure 2. The bakelite block is provided with a circular depression which engages the capillary in the right position. This is then tilted so that the platinum wire extending through the plunger engages between two strips of spring brass above, which serve as the electrical contact and mechanical support. The upper end of the vessel comes automatically to a stop with the vessel in a vertical position and the circuit closed. The same arrangement for determinations at body temperature with the reference electrode and KCl bridge mounted in a thermostat is easily made. It is also very satisfactory to use a thermostat for determinations at room temperature. The temperature of the KCl remains considerably more constant than when exposed directly to the drafts and changes in temperature of the room.

The original sample to be run is taken with a 1 cc. tuberculin syringe graduated in one-hundredths of a cubic centimeter (Becton Dickinson & Co.). The syringe is lubricated with mineral oil, then partly filled with oil and emptied to the exclusion of all air bubbles. The sample is then drawn. If blood is used it is transferred into the sampling tubes of a trifle more than 1 cc. capacity for centrifugation and storage. If cerebro-spinal fluid or other body fluid not requiring centrifugation is used the samples are

stored in the syringes ready for transfer to the electrode. They are protected against loss of carbon dioxide by sealing the needle with oil.

Whole blood is transferred from the syringe to the collection tubes which are partly filled with oil. The oil is displaced from below by the blood. The ground glass stopper which is made of capillary tubing is then inserted which forces most of the oil out through the capillary. The samples are chilled immediately in a small balsa wood ice box and centrifuged as soon as possible. When the blood is centrifuged the oil which has clung to the side of the tube rises to the top in a shallow layer. This small amount of oil cannot absorb much carbon dioxide from the blood and the long capillary of the stopper must effectively prevent diffusion of carbon dioxide. If the blood sample cannot be transferred and centrifuged at once, the needle of the syringe is sealed its entire length with mineral oil. If cerebro-spinal fluid is studied, 0.20 cc. will suffice for about 4 or 5 determinations if transferred to the electrode with care. The certainty of accurate determinations with such minute samples makes the method valuable in such studies as cerebro-spinal fluid acidity where the loss of large amounts of fluid from the fourth ventricle jeopardizes the experiment.

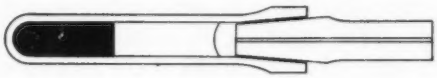

Fig. 3

When the blood sample has been centrifuged the glass stopper is removed with a slow twisting movement permitting the air to enter over the oil through the capillary without extracting carbon dioxide from the sample. The needle of a sampling syringe is then inserted through the oil down to the corpuscles and the plasma slowly withdrawn and the needle sealed with oil. The sample is now ready for transfer to the electrode vessel. This is accomplished with the adapter.

The adapter is a bakelite block attached by thread and screw to a brass bar for mounting on a vertical rod. This block is provided with a tapered hole which accommodates the tapered end of the glass syringe with a minimum of dead space. There is a very slight variation in syringes which must be allowed for in the depth of the tapered hole. This tapered hole is extended horizontally to the other side of the block by a hole of very small bore and capacity. The electrode vessel is mechanically centered over the small opening by means of the yokes which serve as guides. After flushing the adapter with one or more drops of the sample the capillary of the electrode vessel is filled to the air escape. The electrode vessel is then slid downwards off the block. The tip of the capillary is inserted into the KCl

at an angle of about 45° and then brought to the vertical position as described above. These last two precautions avoid trapping of a small bubble of air which might possibly influence the results.

After the determination, which is made in the usual way, the barrel of the electrode vessel is cleaned by means of continuous air suction, by two immersions in distilled water, in alcohol, and in ether and finally dried in the vertical position. The electrode may then be rinsed in a stream of distilled water from a wash bottle and rubbed gently in clean gauze with a twisting motion. By the time the electrode is remoistened and dipped in quinhydrone the barrel is ready for another determination. With an abundance of sample I have flushed the adapter before each determination. This, however, does not seem to be essential. There apparently is little loss of carbon dioxide through the capillary tube of the adapter. The sample which accumulates on the side of the adapter from flushing is conveniently removed with gauze or by short blast of air delivered by a flip of an air cock mounted near the block.

The procedure which has been described has yielded more constant and satisfactory data in less time and with less effort. A number of precise and fatiguing steps have been eliminated or replaced by easier steps. As a net result we have been able to accumulate a larger amount of more dependable data. With the sample ready in the syringe two duplicate determinations including change of samples at the adapter may be made in approximately five minutes. If duplicate determinations do not check three or four determinations may be necessary. On the whole two or three determinations have sufficed.

SUMMARY AND CONCLUSIONS

A transparent bakelite capillary electrode for the determination of hydrogen ion concentration on minute samples is described.

By means of a specially devised bakelite adapter and a 1 cc. syringe samples may be conveniently tranferred without apparent loss of carbon dioxide.

Duplicate determinations and change of samples at the adapter can be made in approximately five minutes.

Samples of cerebro-spinal fluid as small as 0.2 cc. suffice for 4 or 5 determinations.

An improved blood sampling tube for the storage of blood without loss of carbon dioxide was devised.

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The electrode assembly, adapter and sampling tubes may be obtained from Leeds and Northrup Co., 4901 Stenton Avenue, Philadelphia, Pa.

THE REGULATION OF RESPIRATION

XXI. Effects of Intravenous Injection of Sodium Cyanide on the Hydrogen Ion Concentration of the Cerebro-Spinal Fluid of the Dog

ROBERT GESELL and ALRICK B. HERTZMAN

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Assuming that rhythmic discharge of an automatically active neurone is a membrane phenomenon dependent on the composition of the membrane and the composition of the fluid on each side of that membrane we have attempted to accumulate pertinent information on these various factors. Undoubtedly innumerable chemical factors contribute to the behavior of a rhythmically discharging cell, but in the instance of the respiratory center certain factors appear particularly outstanding. Among these factors the acid relations in and about the neuro-respiratory mechanism must surely be included and the state of oxidation of the nerve cell and its membrane should also receive careful consideration.

In earlier papers (Gesell, 1923, 1925; Gesell and Hertzman, 1927) we presented data indicating a parallelism between the magnitude of pulmonary ventilation and intra-cellular acidity and offered this as a probable explanation of increased ventilation produced by the administration of sodium cyanide. If now we assume that the acidity of the cerebro-spinal fluid is indicative of the acidity of the immediate external environment of the respiratory neurone we have the opportunity of studying changes in acidity outside the cells as well. Granting that acidity is the primary variable in the control of ventilation pertinent data might thus be obtainable on which to postulate the relative significance of acidity inside and outside the respiratory neurone.

Figures 1 and 2 represent a selected group of experiments showing effects of 0.003M sodium cyanide in m/6 sodium chloride injected slowly into the femoral vein. The amount injected and the weight of the dog are given on the individual records. The duration of the period of injection and of recovery are obtainable with the use of the five minute scales on each graph. Changes in acidity of the cerebro-spinal fluid were followed with the continuous method in which the manganese dioxide electrode is inserted directly into the fourth ventricle. These changes are shown as continuous curves labeled MnO_2. Downstroke indicates increasing alka-

linity. The procedure for recording acid changes is the same as that previously described (Gesell and Hertzman, 1926a, 1926b). The manganese dioxide acidity curves are checked by pH determinations with the quinhydrone electrode on 0.20 cc. samples drawn at the intervals indicated on the records. The methods of preserving and transferring these samples to the quinhydrone electrode are described in the preceding paper (Gesell, 1928). The curves established by the quinhydrone electrode are plotted on the original records in broken line and labeled Q.H. and the pH values indicated. Attention is called to the fact that no correction factor was determined by comparison with the hydrogen electrode. It is, therefore, possible that all of the absolute values may be slightly more acid as is the case for blood (Cullen and Earle, 1928). Whether any other corrections are necessary was not determined. Mean blood pressure, pulmonary ventilation and changes in oxygen consumption are recorded as in the earlier experiments. Where the chest is intact and the animal controls its ventilation by its own respiratory movements the spirometer record indicates the respiratory effort. Where pneumo-thorax was established and constant artificial ventilation administered abdominal movements are recorded by a vertically moving rod attached by means of thread and pulley to the most mobile part of the abdominal wall.

In figures 1A, B, C and D sodium cyanide was administered to animals with the chest intact. Pulmonary ventilation was consequently variable and under physiological control. Injection invariably augmented both the rate and amplitude of ventilation and the initial period of augmented ventilation was invariably associated with increasing alkalinity. This was most marked during the period of reduced oxidations which is indicated by the gradient of the respiratory record. In figure 1A there was an increase in alkalinity amounting to 0.14 pH. A few minutes after the end of injection when the oxidations increase again there is a distinct change in the acid direction which continues for a long time and to a hydrogen ion concentration considerably greater than in the normal period preceding injection. In figure 1A the cerebro-spinal fluid at the right of the record is 0.3 pH more acid than normal. It increased in acidity from pH of 7.26 to 6.94.

It will be noted that the directional changes in acidity of the cerebro-spinal fluid agree with those obtained on the blood with intravenous injection of sodium cyanide (Gesell and Hertzman, 1927). In those experiments it was suggested that the increased alkalinity of the blood occurring during the markedly increased ventilation of the initial period was probably associated with increased intra-cellular acidity. Without repeating the argument we will assume here too that the period of increasing alkalinity of the cerebro-spinal fluid is accompanied by increasing acidity of the brain. At the close of injection of cyanide we found that the blood turned acid

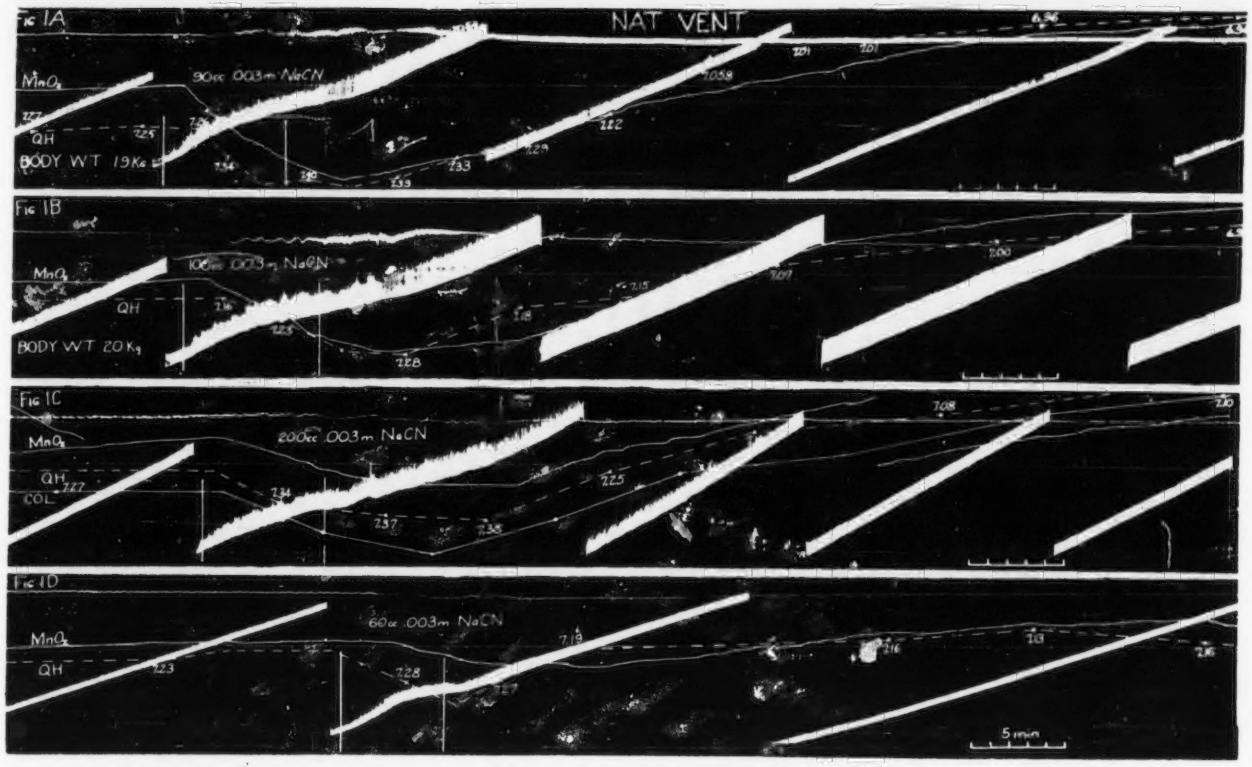
Fig 1A
NAT VENT
MnO2
90cc .003m NaCN
QH
BODY WT 19Kg
Fig 1B
MnO2
100cc .003m NaCN
QH
BODY WT 20 Kg
Fig 1C
MnO2
200cc .003m NaCN
QH
COL
Fig 1D
MnO2
60cc .003m NaCN
QH
5 min

Fig. 1

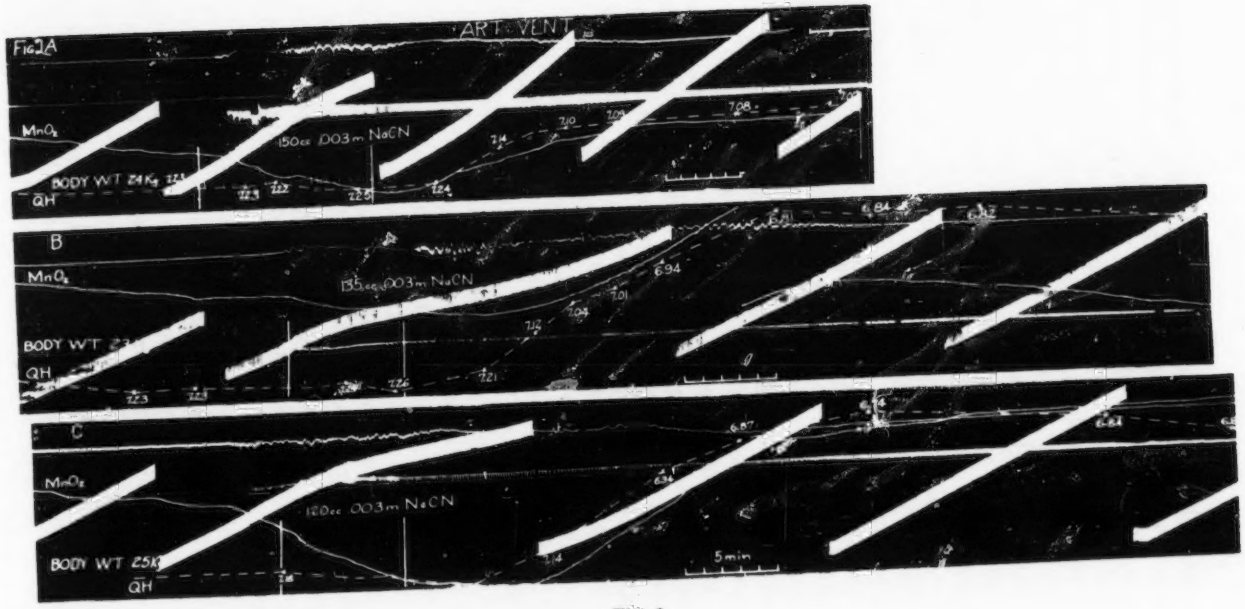
Fig 2A
ART. VENT.
MnO_2
150cc .003m NaCN
BODY WT 24 Kg
QH
B
MnO_2
BODY WT
QH
C
MnO_2
120cc .003m NaCN
BODY WT 25K
QH
5 min

Fig. 2

just as the cerebro-spinal fluid does. The change in acidity is pronounced as oxidations begin and is associated with a marked formation and liberation of carbon dioxide as demonstrated in the expiratory gas experiments (McGinty and Gesell, 1927). The increasing blood acidity is, therefore, most probably due to the simultaneous diffusion of lactic and carbonic acid from the tissues. The cerebro-spinal fluid, however, may be influenced directly by the blood as well as by the tissues. The relative effect of these two factors is difficult to determine. Whatever the relative effect may be the results agree with the probability of the brain turning acid as the cerebro-spinal fluid turns alkaline and a recovery towards normal tissue acidity as the cerebro-spinal fluid turns acid.

In figure 2 in which ventilation remains constant the main difference in the results is the absence of the alkaline dip on the administration of sodium cyanide and the more rapid increase in acidity of the cerebro-spinal fluid during recovery. The same differences hold for the blood experiments where cyanide is injected with physiologically and mechanically controlled ventilation. The absence of the alkaline dip is of course attributable to the absence of increased ventilation which drives the carbon dioxide off. The more rapid acid change is undoubtedly due to the accumulation of carbon dioxide in the tissues during the period of constant ventilation. The respiratory effort during constant ventilation is increased as before. If our reasoning holds that the interior of the cell turns acid with cyanide injection and that the cerebro-spinal fluid represents the immediate external environment then it would appear that increased ventilation resulting from intravenous injection of cyanide was invariably associated with increased intracellular acidity and variably accompanied by increasing, by constant or by decreasing alkalinity of the extra cellular environment. This by no means indicates that extra cellular acidity exerts no effect. As a matter of fact it is possible that increased extra cellular alkalinity of the external environment is stimulating which may agree with the numerable exceptions to the direct relation between blood acidity and pulmonary ventilation which we have repeatedly pointed out. The results of Andrus and Carter (1926) on the heart are also interesting in this connection.

In addition to the differences in acidity of the cerebro-spinal fluid under normal and mechanical control of ventilation there was also a fairly common difference in the respiratory movements under these two conditions. Whereas the progressive administration of cyanide, if it was not too rapid, brought about a progressive increase in ventilation, a similar injection during constant artificial ventilation increased the respiratory movements progressively for a relatively short time, then a tendency towards depression appeared. With the advent of recovery at the end of injection of cyanide the ventilation progressively decreased under normal control.

Under constant mechanical control the respiratory movements frequently showed a secondary augmentation after which they progressively decreased —that is, with constant ventilation there were two maxima of respiratory movements one during the administration of cyanide and another during recovery. The difference in the acidity of the cerebro-spinal fluid during the administration of cyanide might possibly account for the difference in respiratory movements during this period, but during recovery the cerebro-spinal fluid turns distinctly acid under both conditions. Since the supply of oxygen to the tissues during cyanide poisoning is abundant, it is possible that over-accumulation of acid occurring during constantly maintained ventilation may exert a depressing effect upon the activity of the respiratory center and when this excess acid has been removed by a return of normal oxidations a second maximum respiratory effort appears.

It will be noted that our records show a marked acceleration of rate of breathing during the period of disturbed oxidations and that there is some tendency to a slowing of that rate as oxidations improve. This agrees in general with the observations of others on the relative effect of low oxygen and carbon dioxide on the rate of respiratory movements. Though carbon dioxide and low oxygen both accelerate and augment respiratory movement it appears that the low oxygen exerts a comparatively greater effect on rate and carbon dioxide a comparatively greater effect on amplitude. At times, however, these differences are by no means definite. Whether these minor differences are attributable to differences in acidity of the external environment with consequent differences in the stability of the neurone membrane or whether they are due to variation in stability of the neurone membrane due to differences in state of oxidation of the membrane based on supply of oxygen is a matter of speculation. We wish only to call attention now to the effect of variation in pH on a mercury peroxide membrane between mercury and a hydrogen peroxide solution adjusted with acetic acid and sodium acetate. A polarizing current passing through such an inorganic model leads to a rhythmic discharge of the membrane (Bredig and Weinmayer, 1923, and von Antropoff, 1907). This discharge is slowed by a stabilizing effect of increasing hydrogen ion concentration of the peroxide solution. The behavior of this model suggests the advisability of attempting an evaluation of extracellular acidity in all disturbances in pulmonary ventilation. The smaller effect of carbon dioxide on respiratory rhythm might thus be explained.

SUMMARY

The effect of intravenous injection of sodium cyanide on the hydrogen ion concentration of the cerebro-spinal fluid was studied. The changes in acidity were continuously recorded with the manganese dioxide electrode and checked with the quinhydrone electrode.

One set of experiments was performed with the chest intact in which ventilation was under physiological control and a second set in which ventilation was maintained constant by mechanical control.

Under physiological control of ventilation the cerebro-spinal fluid first turned alkaline and during recovery increased in acidity well above normal. Under constant mechanical control the initial alkaline change was virtually missing and the subsequent increasing acidity was more abrupt.

The initial alkalinity under physiological control was attributed to the augmented ventilation which increased the elimination of carbon dioxide. The accelerated acid change with constant ventilation was attributed to a greater accumulation of carbon dioxide during the period of unaugmented ventilation.

Assuming that intra cellular acidity increases as a result of impaired oxidation produced by cyanide and that the acidity of the cerebro spinal fluid represents the acidity of the immediate external environment of the respiratory neurone, increased pulmonary ventilation was associated with increased intracellular acidity but variably accompanied by increasing, by constant or by decreasing alkalinity of the extracellular environment.

The relative significance of intra- and extracellular acidity is discussed.

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THE REGULATION OF RESPIRATION

XXII. Effects of Low Alveolar Oxygen on the Hydrogen Ion Concentration of the Cerebro-Spinal Fluid of the Dog

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This paper deals with effects of administration of gaseous mixtures low in oxygen and of subsequent re-administration of room air on the hydrogen ion concentration of the cerebro-spinal fluid of the dog. The object of the study was to follow changes in acidity produced by impaired oxidations from limitation of oxygen supply and to compare these results with those obtained with impairment of the oxidative mechanism of the cell by injection of sodium cyanide. Results of selected experiments are shown in figures 1 and 2. In figure 1 gaseous mixtures were administered with the chest intact. In figure 2 pneumo-thorax was established and physiological control of ventilation substituted by mechanical ventilation of constant rate and volume.

Inspection of the figures shows similarities in changes in acidity produced by low alveolar oxygen and re-administration of room air when compared with the immediate and late effects of sodium cyanide. In the experiments in which gaseous mixtures low in oxygen were administered during normal physiological control of ventilation the cerebro-spinal fluid turned distinctly alkaline as indicated by the manganese dioxide and quinhydrone electrodes. For example, in figure 1A in which a 6 per cent mixture of oxygen was administered the fluid turned alkaline approximately 0.25 pH and on readministration of room air the hydrogen ion concentration increased rapidly to the pre-administration level. It then increased more slowly to a level slightly above normal. In figure 1B an 11 per cent mixture of oxygen was administered over a longer period. The alkaline change during the administration was less, but the acid change on re-administration of room air was greater. In figure 1C in which a 7 per cent mixture of oxygen was administered for a long time the acid change during recovery was the largest.

In figure 2 in which the gaseous mixtures were administered by artificial ventilation the immediate effects of low alveolar oxygen were less striking than in the experiments in which ventilation was allowed to increase. This

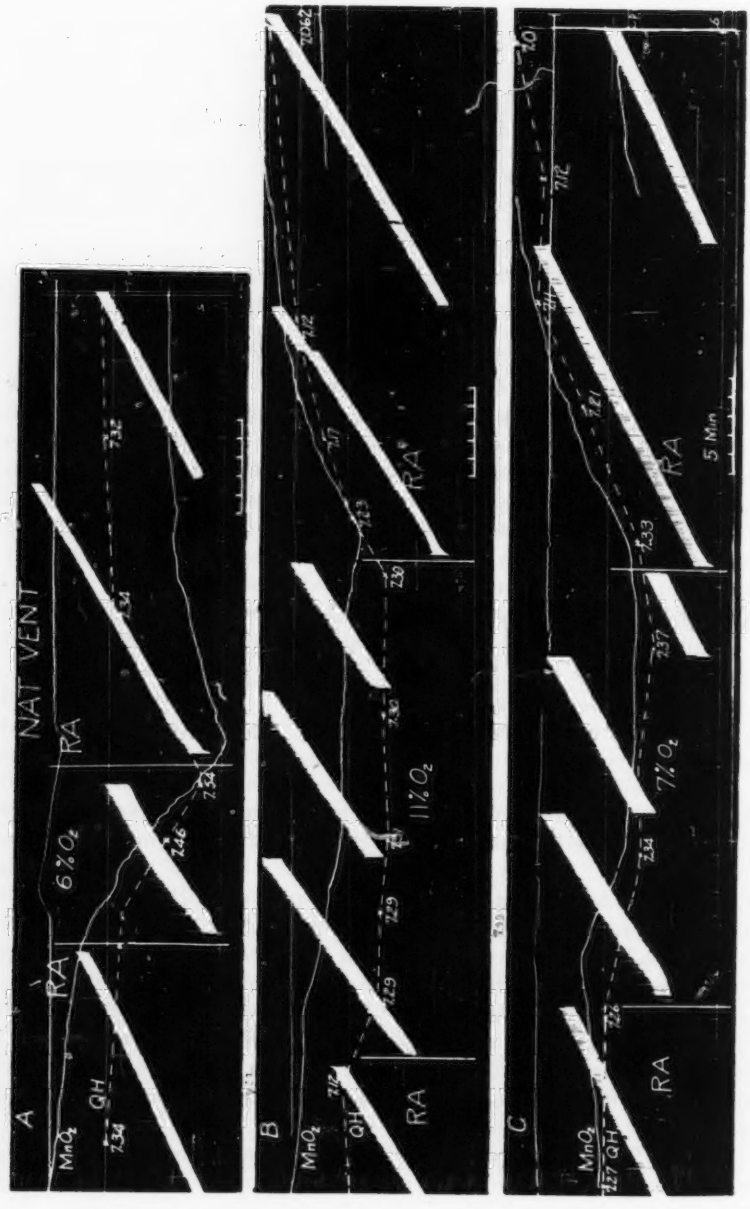
A
NAT VENT
RA
6% O2
MnO2
QH
RA
B
11% O2
MnO2
QH
RA
C
7% O2
MnO2
QH
RA
5 Min

Fig. 1

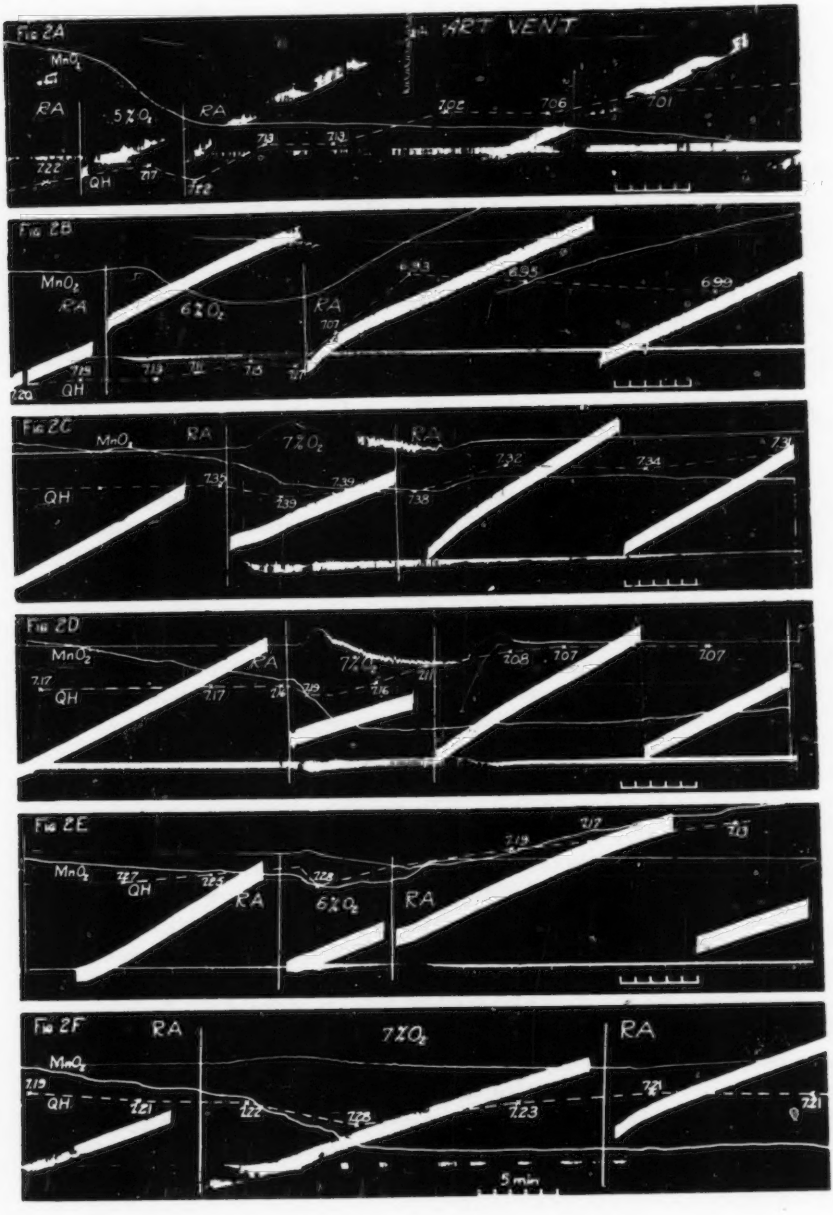
Fig 2A
ART VENT
MnO2
RA
5%O2
RA
7.22
QH
7.17
7.12
7.25
7.06
7.01
Fig 2B
MnO2
RA
6%O2
RA
6.93
6.95
6.99
7.07
7.20
QH
Fig 2C
MnO2
RA
7%O2
RA
7.32
7.34
7.31
QH
7.39
7.39
7.38
Fig 2D
MnO2
RA
7%O2
7.08
7.07
7.07
7.17
QH
7.17
7.16
Fig 2E
MnO2
7.19
7.17
7.13
QH
7.25
7.28
RA
6%O2
RA
Fig 2F
RA
7%O2
RA
MnO2
7.19
QH
7.21
7.22
7.23
7.21
7.21
5 min

Fig. 2

also agrees with the findings in the cyanide experiments. There is, however, a slight difference between the cyanide and low oxygen experiments with constant ventilation. Whereas in the cyanide experiments there was only the faintest indication, if any, of decreased hydrogen ion concentration of the cerebro-spinal fluid, in the low oxygen experiments the indications were more certain. This difference in the cyanide and low oxygen experiments might conceivably be related to the increased alkalinity of the blood produced by a lower state of oxidation of the hemoglobin. With re-administration of room air and return of tissue oxidations the fluid turned acid as in the cyanide experiments.

A comparison of the respiratory records in the cyanide and low oxygen experiments shows similarities in the respiratory movements also indicative of similar chemical changes in the respiratory mechanism. For example, administration of gaseous mixtures low in oxygen during normal control of ventilation produced a long maintained augmentation of ventilation similar to that produced by continued injection of sodium cyanide. On re-administration of room air ventilation gradually diminished as in the cyanide experiments. Likewise when alveolar oxygen was lowered during constant ventilation there was first a decided augmentation of respiratory movements, both in rate and amplitude. This frequently gave way within a minute or more to a slower rate of respiratory movements. In some experiments the amplitude diminished too. Re-administration of room air frequently gave rise to a second augmented respiratory effort as was noted in the cyanide experiments. In some instances this secondary augmentation was followed by a rapid reduction in respiratory movements (see fig. 2F). In other instances the respiratory movements diminished much more slowly (see fig. 2E). In figure 2D the augmented ventilation was well maintained if not increased and in figures 2A and 2B progressive augmentation occurred. These differences in respiratory movements during the period of recovery are most likely due to variations in the effectiveness of the artificial ventilation for any given animal. Unless the volume of ventilation is properly gauged at the outset, respiratory movements will tend either to decrease or increase progressively. Conforming with the effectiveness of ventilation in the various experiments it will be noted that where respiratory movements were definitely increasing prior to administration of low oxygen (figs. 2A, B and D) recovery failed to occur on re-administration of room air. Where respiratory movements increased very slowly before administration of low oxygen (fig. 2E) there was a slight tendency towards recovery on re-administration of room air. In figures 2C and 2F where initial ventilation was most effective recovery was most rapid and complete. In that connection it will also be noted that the final increase in hydrogen ion concentration of the cerebro-spinal fluid was the smallest.

Allowing for the alkaline drift of the manganese dioxide electrode by

extending the normal or pre-administration portion of the curve fairly good agreement between the manganese dioxide and quinhydrone electrodes is indicated. Perhaps the most striking disagreement appears in figure 2B during administration of a 6 per cent mixture of oxygen. During this period the cerebro-spinal fluid according to the quinhydrone electrode turns slowly acid while according to the manganese dioxide electrode it is turning alkaline Granting that the quinhydrone determinations are the more reliable the "alkaline" E. M. F. of the manganese dioxide electrode suggests the appearance of reducing substance from the tissues into the cerebro-spinal fluid. We shall discuss this phase of the subject in greater detail in a later paper.

SUMMARY

Effects of lowered alveolar oxygen pressure followed by re-administration of room air on the hydrogen ion concentration of the cerebro-spinal fluid of the dog were followed with the manganese dioxide and quinhydrone electrodes.

In the intact animal with ventilation under physiological control the administration of gaseous mixtures with low oxygen content was accompanied by a prompt and decided decrease in the hydrogen ion concentration of the cerebro-spinal fluid. Re-administration of room air was followed by an increase in the hydrogen ion concentration exceeding the pre-administration level.

Administration of gaseous mixtures of low oxygen content during artificial ventilation of constant volume was frequently accompanied by a small but definite decreased hydrogen ion concentration of the cerebro-spinal fluid. Re-administration of room air was accompanied by an increased hydrogen ion concentration exceeding the pre-administration level.

The greater initial alkaline change during lowered alveolar oxygen with ventilation under normal control was attributed to the increased ventilation which is absent during artificial ventilation.

Lowered alveolar oxygen with the chest intact elicits a prolonged and progressive augmentation of ventilation. Re-administration of room air was frequently accompanied by progressive recovery.

Lowered alveolar oxygen during pneumothorax and constant ventilation elicited a progressive augmentation of respiratory movements followed by a decline. Re-administration of room air elicited a secondary augmentation frequently followed by recovery.

The chemical and respiratory response to impaired oxidations produced by cyanide and low alveolar oxygen are compared. The relation of intra and extra cellular acidity to respiratory movements showed marked similarities.

THE REGULATION OF RESPIRATION

XXIII. Effects of Hemorrhage and Reinjection on the Hydrogen Ion Concentration of the Cerebro-Spinal Fluid of the Dog

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Effects of hemorrhage and reinjection on the hydrogen ion concentration of the cerebro-spinal fluid of the dog were studied under two conditions. In one set of experiments the animals were intact and ventilation was under physiological control. In the other set pneumothorax was established and ventilation was maintained constant by mechanical control. Three experiments under each of these conditions are shown in figures 1 and 2. In figure 1 ventilation is variable and in figure 2 constant.

The hemorrrhages on the whole were large enough to augment respiratory movements, but never to the same degree noted in the cyanide and low oxygen experiments. The absence of a distinct alkaline change in the cerebro-spinal fluid during hemorrhage with the chest intact may thus be explained. On reinjection of the drawn blood the cerebro-spinal fluid turned acid as in the cyanide and oxygen experiments. The acid changes, however, were relatively small.

With constant ventilation the effects of hemorrhage were more positive. In figure 2A hemorrhage lasted approximately twenty minutes. During this period the hydrogen ion concentration increased steadily from pH of 7.30 to 7.20. Respiratory movements also progressively increased. On reinjection of the blood there was a sudden increase in acidity of the cerebro-spinal fluid from pH 7.20 to 7.06 accompanied by correspondingly augmented respiratory movements. Respiratory movements and hydrogen ion concentration then diminished very gradually. At the end of the record both the acidity of the cerebro-spinal fluid and the respiratory movements were still above normal. The impaired oxidation during hemorrhage accompanied by increased acidity of the cerebro-spinal fluid is indicative of increased anaerobic acid metabolism. The hyperoxidation following reinjection confirms the findings of the expired gas experiments (McGinty and Gesell) in which it was also found that carbon dioxide pressure of the alveolar air is markedly increased. The increased carbon dioxide formation coupled

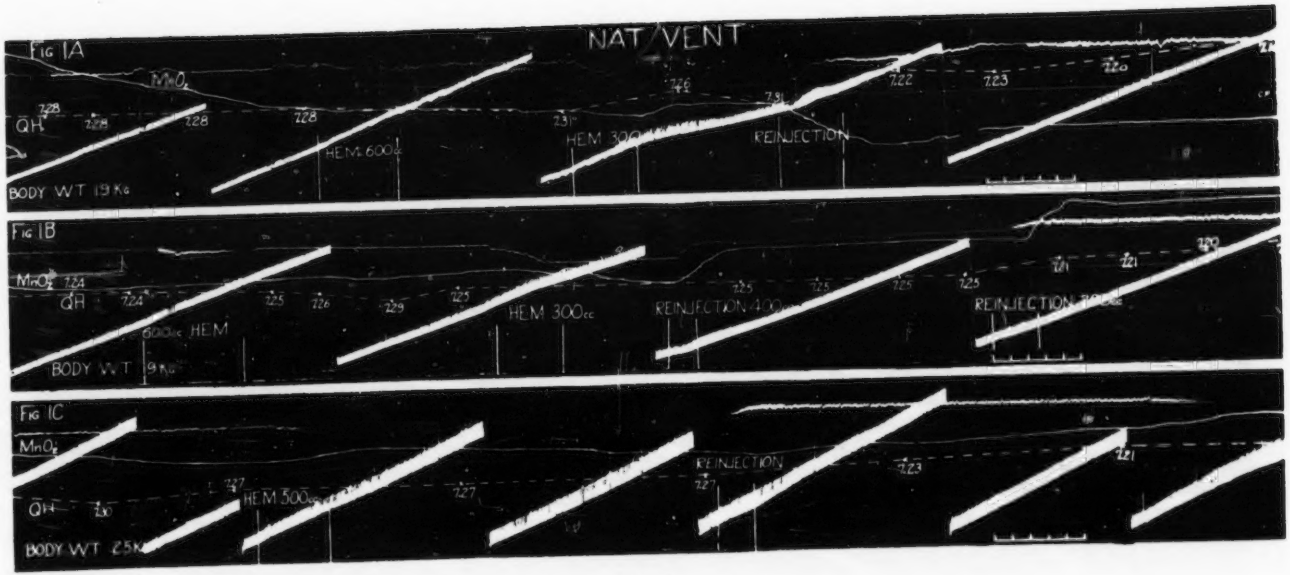
NAT VENT
Fig 1A
MnO_2
QH
HEM 600cc
HEM 300cc
REINJECTION
BODY WT 19 Kg
Fig 1B
MnO_2
QH
600cc HEM
HEM 300cc
REINJECTION 400cc
REINJECTION
BODY WT
Fig 1C
MnO_2
QH
HEM 500cc
REINJECTION
BODY WT 25K

Fig. 1

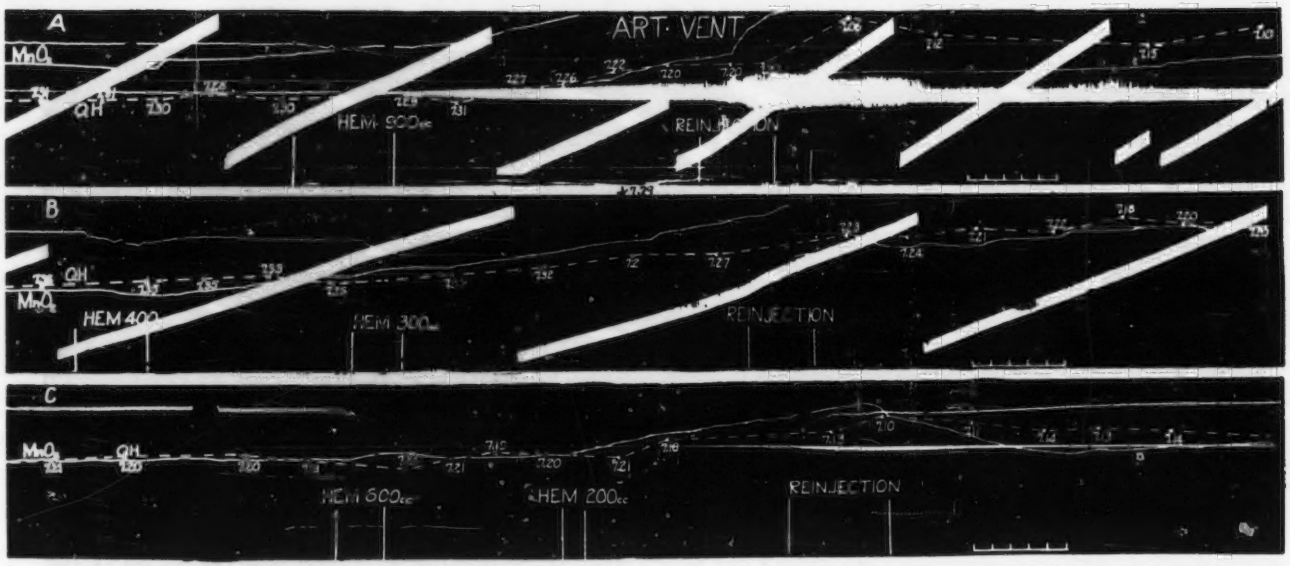
A
ART. VENT.
MnO2
QH
HEM. 500cc
REINJECTION
B
QH
MnO2
HEM 400
HEM 300
REINJECTION
C
MnO2
QH
HEM 600cc
HEM 200cc
REINJECTION

Fig. 2

with the effects of the anaerobic acids formed during hemorrhage most probably account for the final increase in acidity of the cerebro-spinal fluid. In figure 2B hemorrhage was carried out in two steps. The respiratory movements were not recorded. The acid changes and their relation to oxidations are similar to, though not quite as striking as those in figure 2A. In figure 2C the typical results of figure 2A are shown again. After the first hemorrhage the initial alkaline change gave way to the prehemorrhage hydrogen ion concentration. There was no change in respiratory movements. After the second hemorrhage there was a gradual increased acidity from pH 7.20 to about 7.15. This increase was associated with a progressive increase in respiratory movements. On reinjection the sharply increased acidity of the cerebro-spinal fluid followed by a gradual decrease in hydrogen ion concentration was accompanied by a corresponding increase and decrease in respiratory movements. Again as in figure 2A there was a marked parallelism between hydrogen ion concentration and respiratory movements. At the end of the record the hydrogen ion concentration of the cerebro-spinal fluid and the amplitude of respiratory movements are both above the prehemorrhage values.

SUMMARY

A limited number of experiments on the effect of hemorrhage on the acidity of the cerebro-spinal fluid of the dog is reported.

With the chest intact and ventilation under normal control hemorrhage produced no or only a small change in acidity of the cerebro-spinal fluid. Reinjection of the blood always increased the hydrogen ion concentration.

During pneumothorax and constant ventilation hemorrhage increased the acidity of the cerebro-spinal fluid. Reinjection produced a further increase which was followed by recovery of varying degrees.

A directional relation between the hydrogen ion concentration of the cerebro-spinal fluid and respiratory movements obtained.

THE REGULATION OF RESPIRATION

XXIV. A Comparison of the Effects of Mechanical Asphyxia with the Lungs Filled with Room Air and with Oxygen on the Hydrogen Ion Concentration of the Cerebro-Spinal Fluid of the Dog

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In an earlier paper (Gesell and Hertzman, 1926) we reported the effects of mechanical asphyxia produced by interrupting uniform ventilation during pneumothorax. If the periods of asphyxia were equal it was found that interruption of ventilation when the lungs were filled with oxygen increased the hydrogen ion concentration of the arterial blood considerably more than when the lungs were filled with room air. Respiratory movements on the other hand were greater during asphyxia when the lungs were filled with room air. These results were checked with the hydrogen electrode. The findings thus established an important exception to the prevailing theory of the control of ventilation by the hydrogen ion concentration of the arterial blood. Though it is admitted that the difference in stimulation of respiratory movements may be an effect of oxidations independent of acid changes arguments were presented indicating a possible parallelism between the degree of excitation and the acidity of the respiratory center. The findings were thus aligned with this central acid theory of control of ventilation as opposed to the blood acidity theory of control. Further confirmatory evidence of the relation of ventilation to the acidity of the respiratory center was sought in the cerebro-spinal fluid experiments.

A record of the effects of mechanical asphyxia of 150 seconds duration on mean blood pressure, hydrogen ion concentration of the cerebro-spinal fluid, and respiratory movements is shown in figure 1. In the upper record ventilation was suspended during the administration of room air and in the lower record during the administration of pure oxygen. The differences were definite. When ventilation was stopped during the administration of room air blood pressure increased and respiratory movements were markedly stimulated. With readministration of room air the blood pressure rapidly returned to normal values and respiration subsided. The

changes in the hydrogen ion concentration as recorded with the manganese dioxide electrode were very slow. The acidity began to increase only towards the close of asphyxia. The increase was very gradual and persisted about seven minutes and then slowly decreased towards normal for another period of approximately eight or nine minutes.

In the lower record where ventilation was interrupted while administering pure oxygen there was no visible increase in mean blood pressure. Note that the pump was stopped at the end of inspiration in both ob-

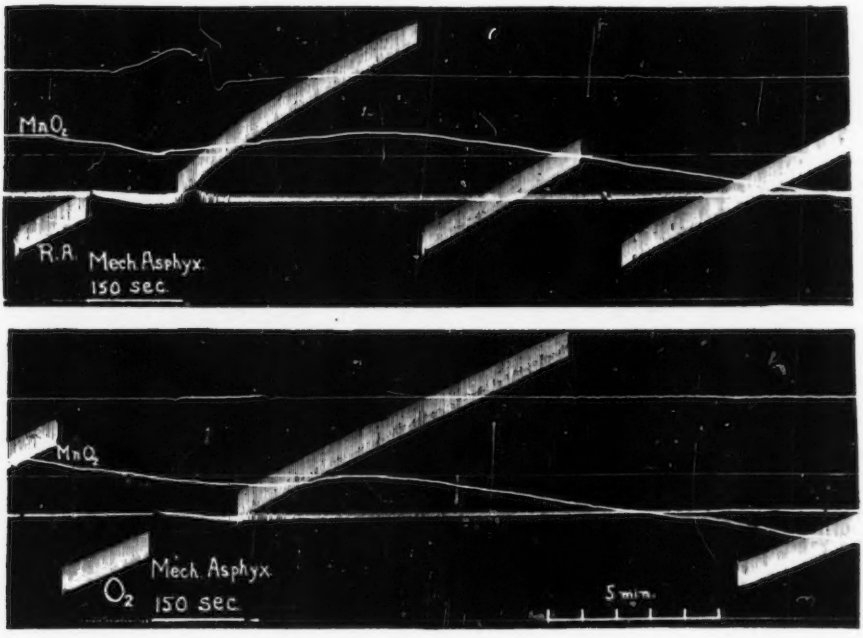

Fig. 1

servations. The difference in the response of the circulatory and respiratory systems, therefore, cannot be accounted for by a difference in phase of interruption of ventilation. The respiratory movements were stimulated by asphyxia but to a much less degree than in the preceding experiment. On reëstablishment of ventilation the rapid respiratory movements give way as in room air asphyxia to slower movements synchronous with the pump. The amplitude, however, is considerably augmented. This increased amplitude then gradually diminishes to the pre-asphyxial amplitude. Corresponding to the relatively smaller stimulation of the

circulatory system and respiratory movements in oxygen asphyxia there was smaller increase in hydrogen ion concentration of the cerebro-spinal fluid. All indications are that the tissues suffered greater chemical change by asphyxia interrupting administration of room air than by asphyxia with lungs filled with oxygen. The problem is to determine the most pertinent changes influencing respiratory movements.

The most obvious changes are the disturbances in the acid base equilibria and the disturbances in oxidations which have given rise to these altered acid base equilibria. If we can accept the manganese dioxide acidity curves we are inclined to conclude that asphyxia with the lungs filled with room air gave rise to a greater disturbance of the acid base equilibrium of the tissues in the acid direction than did similar asphyxia with abundance of oxygen. But if we compare the changes in acidity of the blood under similar circumstances we might conclude the reverse for room air asphyxia produced a smaller increase in blood acidity than did oxygen asphyxia. It would thus appear that the changes in acidity of the blood and of the cerebro-spinal fluid are of a somewhat different nature. In that connection it is pertinent to compare the time relation of the acid changes in the blood and in the cerebro-spinal fluid. In the blood the increase of acidity comes on promptly with either room air or oxygen asphyxia but in oxygen asphyxia the increase is greater and more rapid. When artificial ventilation is reëstablished the decreased hydrogen ion concentration is also immediate. In the cerebro-spinal fluid the increase and decrease of the hydrogen ion concentration is markedly delayed. The hydrogen ion concentration of the cerebro-spinal fluid increases as the hydrogen ion concentration of the blood decreases. Presumably the rapid changes in acidity of the blood are related primarily to the degree of oxidation of hemoglobin and to the carbon dioxide pressure of the blood. These are factors susceptible of rapid change. Possibly the changes in acidity of the cerebro-spinal fluid are related to diffusion of more slowly moving members of the tissue buffer systems, lactic acid for example.

Analysis of the ventilation record indicates that during room air asphyxia a deficit of oxygen developed for the consumption of oxygen at the close of asphyxia remains above normal for several minutes. In the oxygen experiment there is very little change in the basal metabolic gradient indicating little or no change in the rate of oxidations from mechanical asphyxia. Two assumptions seem reasonably certain—lactic acid most probably forms and accumulates to a greater degree in room air asphyxia than in oxygen asphyxia; the second assumption that the elimination of carbon dioxide is hampered with a lowered alveolar oxygen pressure by a disturbance in the dual function of hemoglobin is upheld by the expired gas experiments (Gesell and McGinty, 1927). The smaller acid change in the cerebro-spinal fluid in oxygen asphyxia

would, therefore, agree with a smaller formation of fixed acids in the tissues and with a better transport of carbon dioxide from the tissues to the lungs.

Despite the agreement of the manganese dioxide electrode curves and the expected changes in acid equilibria within the tissues we preferred to check our results with the quinhydrone electrode. Simple as these experiments may seem they taxed our patience and time most severely. Our major difficulty arose from the fact that we were comparing effects of a minor order of only a few hundredths of a pH separated by long intervals of time in a highly unstable system. It is, therefore, necessary not only to establish a greater increase in acidity with room air asphyxia before oxygen asphyxia but after it as well. This has been done with the manganese dioxide electrode in four experiments. Though some of the quinhydrone determinations supported the findings with the manganese dioxide electrode they were too irregular to be satisfactory. The slight effects of withdrawing samples on the acidity of the cerebro-spinal fluid were sufficient to discourage further efforts to clinch the findings with this more reliable electrode.

In this connection attention should be called to the behavior of the manganese dioxide electrode in the cerebro-spinal fluid. Not infrequently mechanical asphyxia produced an apparent "alkaline" E.M.F. while in other animals this effect was entirely missing. On the whole it was more difficult to use the electrode in the fourth ventricle than in the blood stream. While it is frequently possible to use a manganese dioxide electrode for several experiments in the blood stream, in many animals the manganese dioxide may be completely dissolved within an hour if placed in the fourth ventricle. In some experiments we have used as many as three and four very heavily plated electrodes. This rapid solution of manganese dioxide indicated the presence of highly reducing substances in the cerebro-spinal fluid which are relatively less effective or less concentrated in the blood. The interesting fact is that electrodes should behave so differently in different animals. We gained the impression that the diet of our animals was in some way related to the peculiar behavior of the electrodes. At least substituting a meat diet for dog biscuits appeared to diminish the "alkaline" effects of mechanical asphyxia. We have tentatively concluded that mechanical asphyxia leads to frequent appearance of reducing substances in the cerebro-spinal fluid sufficient to oppose the change in E.M.F. produced by increased hydrogen ion concentration. We have also noted an "alkaline" change in E.M.F. on the administration of carbon dioxide. This presumably may be accounted for in the same way. The appearance of reducing substances in the circulating blood and cerebro-spinal fluid during augmented respiratory movements naturally suggests the possibility of a causal connection. This phase of the subject, however, requires special study.

SUMMARY

A comparison of effects of mechanical asphyxia with the lungs filled with room air and with oxygen on the hydrogen ion concentration of the cerebro-spinal fluid of the dog was made.

As indicated by continuous records obtained with the manganese dioxide electrode, mechanical asphyxia with the lungs filled with room air increased the acidity most.

Since the increase in acidity of the blood is greater with mechanical asphyxia when the lungs are filled with oxygen it is suggested that the greater change in acidity of the cerebro-spinal fluid may be related to the greater formation of lactic acid, consequent on the greater impairment of oxidations.

The results thus support the view that the brain turns more acid during mechanical asphyxia when the lungs are filled with room air even though the blood may turn less acid. The greater stimulation of respiratory movements resulting from the greater impairment of oxidations may consequently be accounted for on an acid basis.

The significance of the appearance of reducing substances in the cerebro-spinal fluid as a result of mechanical asphyxia is discussed.

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STUDIES ON SUPRARENAL INSUFFICIENCY

II. The Relative Importance of Cortex and Medulla in the Susceptibility to Histamine of Suprarenalectomized Rats

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Albino rats, suffering from suprarenal insufficiency, are more susceptible than normal rats to a variety of toxic agents. One of these toxic agents is histamine. Crivellari (1927) showed that suprarenalectomized rats are 12.5 times more susceptible to histamine phosphate than are normal rats. Marmorston-Gottesman and Gottesman (1928) found that their suprarenalectomized rats were about twenty times more susceptible to histamine acid phosphate than were normal rats. Scott (1928) found that most doubly suprarenalectomized rats are killed by doses that produce only transient symptoms in operated control rats, that is, by doses of 10 mgm. of histamine acid phosphate per 100 grams of body weight, if given in the second week after operation. Scott, moreover, deduced from a correlation of various data that the function involved in this diminished resistance to histamine is cortical and is not related to adrenalin deficiency. He proposed this susceptibility to histamine as a functional test for deficient cortex function.

In some previous work I had noticed that occasionally a rat, which had survived a large dose of diphtheria toxin because of the presence of gross accessory cortical tissue, was killed by a small dose of histamine. This led to the investigation of the relative importance of the cortex and of the the medulla of the suprarenal gland in the increased susceptibility to histamine poisoning in suprarenalectomized rats. The results of this investigation follow.

Methods. A mixed stock of colored varieties of the albino rat was used. The selection of animals for experimentation, care of the animal room, diet, after-care, and the technique of suprarenalectomy and control operations have been described in previous papers from this laboratory (Belding and Wyman, 1926; Wyman, 1928a). The histamine used was ergamine acid phosphate (Burroughs Wellcome and Co.). Intraperitoneal injections of a solution containing 50 mgm. of this compound per cubic centimeter of sterile distilled water were used in all cases. All the dosages

are reported as milligrams of ergamine acid phosphate per 100 grams of body weight of the animal.

Normal and control rats. A large amount of histamine is required to kill a normal rat. The intravenous minimal lethal dose of histamine acid phosphate for normal rats was found to be 90 mgm. per 100 grams body weight by Voegtlin and Dyer (1925), from 50 to 70 mgm. per 100 grams by Crivellari (1927), and about 160 mgm. per 100 gram by Marmorston-Gottesman and Gottesman (1928). Scott (1928) found that his operated control rats survived doses of histamine up to 110 mgm. per 100 grams. Because of the expense of large amounts of histamine I did not determine the minimal lethal dose for normal rats. In fifteen cases young rats, about one month of age, survived intraperitoneal doses up to 100 mgm. per 100 grams of body weight. Six rats in which one suprarenal had been removed and the other exposed and handled, and two in which both glands had been exposed and a piece of fat only removed from each suprarenal site, survived intraperitoneal doses of histamine up to 70 mgm. per 100 grams. The injections were given from 10 to 14 days after operation. In the case of the larger doses (50 to 70 mgm. per 100 grams) the respiration was rapid and labored and sometimes the animal was prostrated for a time, but complete recovery from these effects took place in from three to six hours. In the case of the smaller doses (10 to 40 mgm. per 100 grams) the respiration was rapid but the animals were not prostrated and appeared to be fully recovered in two or three hours. In some cases small doses (10 to 20 mgm. per 100 grams) did not visibly affect the animals at all. Evidently single suprarenalectomy or a blank operation does not increase the susceptibility of the rat to histamine.

Suprarenalectomized rats. Fifty-seven doubly suprarenalectomized rats were given intraperitoneal injections of a solution of histamine acid phosphate. The individual doses ranged from 5 to 20 mgm. of histamine per 100 grams of body weight. The minimal lethal dose of histamine which could be depended on to kill a doubly suprarenalectomized rat was found to be 7 mgm. per 100 grams, although of seven rats which were given 5 mgm. per 100 grams, six were killed by this dose while one recovered and was killed later by a dose of 10 mgm. per 100 grams. Of these fifty-seven rats, six were killed by doses of 5 mgm., seventeen by doses of 7 mgm., twenty by doses of 10 mgm., and five by doses of 20 mgm. per 100 grams. Of the other nine rats, five survived doses of 10 mgm., three survived doses of 15 mgm., and one survived a dose of 20 mgm. per 100 grams. The autopsy findings in these nine rats will be described later.

The times between the injections and the death of the animals were fairly constant. In the case of the six rats which were killed by doses of 5 mgm. of histamine per 100 grams, these times ranged from 30 minutes to 5 hours and 7 minutes, averaging 2 hours and 19 minutes. In the case of

the thirty-seven rats which were killed by doses of 7 and 10 mgm., the time from injection to death was between 5 and 6 hours in four cases only. In thirty-three cases the average time was 61.2 minutes, being less than 30 minutes in ten cases, between 30 minutes and one hour in twelve cases, and between one and three hours in eleven cases. No difference was observed in the average period of survival between those receiving 7 mgm. and those receiving 10 mgm. per 100 grams. As a general statement it could be said that, in the case of doubly suprarenalectomized rats, death may be expected within an hour after an injection of 7 or 10 mgm. of histamine per 100 grams of body weight. In the case of five rats which were killed by doses of 20 mgm. per 100 grams, the period of survival averaged 31.2 minutes, ranging from 21 to 46 minutes.

Of the rats which were killed by doses of from 5 to 10 mgm. per 100 grams, six were injected during the second week, sixteen during the second two weeks, six during the second month, seven during the third month, seven during the fourth month, and one during the fifth month after operation. Thus an opportunity was given for observing the results of the test on rats in various stages of suprarenal insufficiency, acute, subacute and chronic. No differences were observed in these six groups of rats with respect to susceptibility or period of survival. The rats in all groups were killed with equal readiness by doses of 7 mgm. or 10 mgm. of histamine per 100 grams. The period of survival averaged in the neighborhood of one hour for each group, and there was a striking similarity in the six series of individual times, both when they were compared with one another and when one was compared with the six series as a whole. Evidently the increased susceptibility of rats to histamine poisoning after double suprarenalectomy is constant, at least up to five months after operation, and does not decrease as the period of survival increases and accessory cortical tissue becomes established.

The changes in the weights of the rats from the time of operation to the time of injection were the same as those observed in any series of suprarenalectomized rats. Of twenty-seven which were injected during the first two months after operation, twenty had lost 2 to 70 grams and seven had gained from 1 to 20 grams. Of fifteen which were injected during the following three months, twelve had gained from 7 to 70 grams and three had lost from 11 to 48 grams. The change in weight is probably correlated with the degree of insufficiency, the greater number of increases in weight in the second group being associated with chronic insufficiency or the establishment of accessory suprarenal masses as functional tissue. No correlation was seen between the weight changes and the susceptibility to histamine poisoning. All were equally susceptible.

Of the forty-two doubly suprarenalectomized rats which were killed by doses of from 7 to 20 mgm. of histamine per 100 grams, fifteen were found at

autopsy to have gross accessory cortical masses in or near the suprarenal sites, and two had masses which were regarded as doubtful accessories. All suspected accessory tissue was fixed in Zenker's or Müller's fluid and carefully examined microscopically. The fifteen cortical masses appeared to be composed of healthy cortical cells, well packed with lipoid material, and all were well organized, enclosed in capsules, and extensively vascularized. As to size, one of these masses was 1 mm. in diameter, seven were from 2 to 3 mm. in diameter, five were from 4 to 5 mm. in diameter, one was 6 by 2 mm., and one was a large irregular mass 12 mm. long and 2.5 mm. thick. Three of the rats had two or three such masses. The average size of one normal suprarenal gland found by actual measurement at operation of numerous glands from these same animals was from 3.5 to 4 mm. in the longest diameter. Most of the fourteen rats had, therefore, fully as much cortical tissue as normal animals. As to dosage, four which received 7 mgm. per 100 grams, seven which received 10 mgm., and four which received 20 mgm. had gross accessory cortical masses. No signs of accessory medullary tissue could be found in any of the rats which were killed by small doses of histamine.

It is known that rats suffering from suprarenal insufficiency are between two and three times as susceptible as normal rats to diphtheria toxin (Belding and Wyman, 1926). The lethal dose of the lot of diphtheria toxin used in this research, for suprarenalectomized rats, averaged 2.75 M.L.D. per gram of body weight (Wyman, 1928b). There is evidence that it is the lack of the cortical portion of the suprarenal that is responsible for the increased susceptibility to diphtheria toxin. In my experience, suprarenalectomized rats possessing gross accessory cortical tissue have not been killed by doses which are fatal for those having no accessory cortical tissue. Four rats, two of which were killed by 7 mgm. of histamine per 100 grams and two which were killed by 10 mgm. per 100 grams, had, a short time previously, survived two doses of diphtheria toxin each. The doses were as follows: 2 and 3 M.L.D. per gram, 2.75 and 4 M.L.D. per gram, two doses of 3 M.L.D. per gram and 2.5 and 4 M.L.D. per gram. Three of these rats were among those having gross accessory cortical tissue. If these rats survived the doses of diphtheria toxin, lethal for rats with cortical insufficiency, because of the presence of accessory cortical tissue, the fact that they were killed by small doses of histamine indicates that the presence of functional cortical tissue alone does not protect against this poison.

Nine of the female rats which were killed by small doses of histamine and which were found to have gross accessory cortical tissue had previously been exposed to males after suprarenalectomy, and had become pregnant and delivered normal litters of from four to six young. This is further evidence that these animals were not suffering from any great degree of cortical insufficiency (Wyman, 1928a).

Of the nine suprarenalectomized rats which survived the injections of histamine, eight were found at autopsy to have gross accessory suprarenal masses in the suprarenal sites, ranging in size from 2 to 7 mm. in diameter. One had four such masses, from 1 to 3 mm. in diameter, but unfortunately no sections were made for microscopic study. Three rats had structures in one suprarenal site which were of the size, shape and color in the gross of normal suprarenal glands. In section two of these were seen to be composed of healthy looking cortical cells arranged as in a normal suprarenal cortex, but no medullary tissue was identified, the sections appearing like those made to one side of the medulla of a normal gland. The other appeared like a normal gland in section and the center was fragmented, as if medullary tissue might once have been present. Two rats had smaller masses, 2 and 2.5 mm. in diameter. In each of these a portion of the mass was composed of cortical tissue and a smaller portion was composed of a different kind of tissue made up of more irregular cells which reminded one of medullary cells. In one case an adjacent mass of tissue contained a quantity of amorphous material stained a deep yellow. One rat had a mass, 2 by 3 mm., about a quarter of which was composed of medullary tissue with a brilliant chromaffin stain and with the tissue spaces packed with amorphous yellow material. One rat had a large mass, 7 by 4 by 3 mm., which looked like a normal gland in the gross, and which was composed of cortical tissue arranged as in a normal gland interspersed with numerous, yellow staining chromaffin cells. Thus of the seven rats which survived the histamine and in which microscopic examination was made of the accessory tissue found at autopsy, two had identifiable chromaffin tissue and seven gave the appearance of having medullary tissue as well as cortical tissue. It must be remembered that it is difficult to identify medullary tissue microscopically unless a good chromaffin stain is obtained.

Three of these surviving rats which had gross accessory suprarenal tissue had previously survived doses of diphtheria toxin lethal for cortically insufficient rats (2.75, 3 and 3.5 M.L.D. per gram), and four others had become pregnant after suprarenalectomy and delivered normal litters of six or seven young. This indicates that the accessory cortical tissue was normally functional.

Autoplastic cortical transplants. Further proof that the increase in susceptibility to histamine of suprarenalectomized rats is the same in animals with and in those without functioning cortical tissue was sought in a study of autoplastic suprarenal transplants. Jaffe and Plavska (1926) and Jaffe (1926) have demonstrated that successful, functioning, autoplastic cortical transplants may be made in rats. They removed both glands in one sitting, transplanted them into four sites in the abdominal muscles, and reported that the cortical tissue regenerated but that the medullary tissue did not regenerate. Using Jaffe and Plavska's technique,

autoplastic transplants of both suprarenal glands were made in a series of eighteen rats between one and a half and two months of age. Successful takes were obtained in twelve of these rats. Injections of histamine were given to these twelve rats, from one to two months after operation. Seven were killed by doses of 10 mgm. of histamine per 100 grams. In four of these the survival time after injection was about 30 minutes, in one it was 57 minutes, and in two it was 134 and 153 minutes. One was killed by 15 mgm. per 100 grams, in 9 minutes. Three were killed by 20 mgm. per 100 grams, the survival times being 27, 31 and 180 minutes. One survived an injection of 20 mgm. per 100 grams and was later killed with ether for autopsy.

Evidence that the transplanted cortical tissue was functioning was afforded by the fact that six of the rats which were killed by histamine had previously survived doses of diphtheria toxin, each dose being larger than the lethal dose for a rat with cortical insufficiency. The doses were 3, 4 and 5 M.L.D. per gram of body weight, in two cases each. The diphtheria toxin was injected 43 days after operation and from 18 to 25 days before the histamine was injected.

All twelve rats were in good condition when the histamine was injected, showing no clinical signs of cortical insufficiency. They had grown as well as normal rats, having gained from 92 to 221 grams apiece, with an average gain of 132 grams apiece for the group. At autopsy they were found to be well supplied with fat. It is known that in rats fat rapidly disappears in cortical insufficiency. One transplant had regenerated in four cases, two had regenerated in five cases, three had regenerated in two cases, and in one case all four had regenerated. In addition, in four cases one or two gross cortical accessories were found in the suprarenal sites, ranging from 1 to 4 mm. in diameter. The size of the transplants varied from 2 to 5 mm. in diameter. In those with one transplant it was 4 or 5 mm. in diameter. In the others at least one transplant was 3 or 4 mm. in diameter. In several cases a single transplant was as large as or larger than a normal suprarenal gland. Thus each of these rats possessed nearly as much or more cortical material than a normal animal. Microscopic examination showed that the transplants were composed of solid masses of cortical tissue surrounded by a capsule. In some cases there were several lobes of encapsulated tissue in a single mass. The cells appeared healthy, well packed with lipoids and normal in every way. The transplants were well vascularized, containing many blood vessels filled with normal blood cells. In the gross an extensive vascularization could be seen in each case, many large and small blood vessels entering or leaving the transplants. The muscular tissue immediately surrounding the transplants was healthy and appeared perfectly normal in section. In only two cases was there any evidence of medullary tissue having survived in the transplants. In

these there was a small group of irregular cells taking the chromaffin stain at one side of one of the masses of transplanted cortical tissue. Both of these were from rats which had survived doses of 10 mgm. of histamine per 100 grams but which had been killed by doses of 20 mgm. per 100 grams. One survived 180 minutes after the injection. In view of the fact that it is said that the medulla never regenerates in a transplant, more investigation on this point seems to be necessary. The one rat which survived an injection of 20 mgm. per 100 grams had one transplant and two large masses of accessory cortical tissue in the suprarenal sites. No medullary tissue could be found in this animal. The reason for survival is unknown.

It appears that in suprarenalectomized rats, with autoplastic cortical transplants equal in bulk to the amount of cortical tissue which normal rats possess, and not suffering from cortical insufficiency as shown by their condition and the tests with diphtheria toxin, the increase in susceptibility to histamine poisoning is the same as that in rats having no cortical tissue and suffering from cortical insufficiency.

Adrenalin and histamine. An attempt was made to protect suprarenalectomized rats from the fatal effects of histamine by injecting adrenalin previous to the injection of histamine. Adrenalin chloride solution (Parke, Davis & Company) freshly diluted in sterile saline solution was used in all cases. The adrenalin solution was injected first and 10 or 15 minutes later the histamine solution was injected. In some cases one or two additional doses of adrenalin of the same strength and quantity as the first dose were given at three hour intervals. Such tests were carried out in 34 doubly suprarenalectomized rats, from 22 to 56 days after operation. The results are given in table 1. Subcutaneous injection of adrenalin solution did not protect the rats from intraperitoneal injection of histamine solution. Intraperitoneal injection of adrenalin solution did afford partial or complete protection from the fatal effects of 10 or 20 mgm. of histamine per 100 grams in more than two-thirds of the cases tried. In table 1 all of the injections of adrenalin are intraperitoneal with the exception of the first two cases (nos. 70 and 80).

Following the injection of 1 or 2 cc. of adrenalin chloride, 1:10,000, and histamine, 10 mgm. per 100 grams, three rats survived and one died in about 20 hours. As stated above, the death of a suprarenalectomized rat may be expected within one hour after the injection of this amount of histamine. A few days later the survivors died in about half an hour after the injection of similar or smaller doses of histamine (7 and 10 mgm. per 100 grams) without the previous injection of adrenalin. No gross accessory suprarenal tissue was found at autopsy. Following the injection of 1 or 2 cc. of adrenalin, 1:50,000, (3 injections 3 hours apart in 3 cases) and 10 or 20 mgm. of histamine per 100 grams, three rats survived and one died in about 24 hours. Five days later two of the survivers were

TABLE 1

ADRENALIN AND HISTAMINE INJECTIONS						HISTAMINE INJECTIONS WITHOUT ADRENALIN			
Rat number	Days after operation	Dilution of adrenalin	Dose of adrenalin	Dose of histamine per 100 grams	Result and survival time in minutes	Days after first dose	Dose per 100 grams	Result and survival time in minutes	Gross accessory cortex
				mgm.					
70	22	1:10,000	0.5 cc. subcutaneously	10	Died 17				0
80	22	1:10,000	1 cc. subcutaneously	10	Died 33				0
79	22	1:10,000	0.5 cc.	10	Died 20 hours				0
75	23	1:10,000	0.8 cc.	10	Survived	2	10 mgm.	Died 31	0
71	23	1:10,000	1 cc.	10	Died 20 hours				0
78	22	1:10,000	1 cc.	10	Survived	3	10 mgm.	Died 26	0
87	27	1:10,000	2 cc.	10	Survived	4	7 mgm.	Died 31	0
90	24	1:50,000	0.5 cc.	10	Died 21				0
10	23	1:50,000	1 cc.	10	Died 24 hours				0
95	27	1:50,000	1 cc.	20	Died 77				0
94	26	1:50,000	1 cc. 3 injections	10	Survived	5	7 mgm.	Died 17	0
92	26	1:50,000	2 cc. 3 injections	10	Survived	5	7 mgm.	Died 26	0
96	27	1:50,000	2 cc. 3 injections	20	Survived				0
35	32	1:100,000	2 cc.	10	Died 98				0
399	30	1:100,000	1 cc. 3 injections	10	Died 9 hours				1
88	24	1:100,000	2 cc.	10	Died 106				0
38	51	1:100,000	2 cc. 2 injections	10	Died 338				0
25	56	1:100,000	2 cc. 3 injections	10	Survived	2	10 mgm..	Died 61	1
8	56	1:100,000	2 cc. 3 injections	20	Survived	2	20 mgm.	Died 130	0
85	25	1:100,000	2 cc.	30	Died 25				0
7	56	1:100,000	2 cc.	30	Died 20				0
1	56	1:100,000	2 cc.	30	Died 144				1
61	25	1:100,000	2 cc.	40	Died 40				0
52	45	1:100,000	2 cc.	40	Died 49				0

37	51	1:100,000	2	cc.	40	Died 82				0
40	51	1:100,000	2	cc.	50	Died 88				0
39	51	1:100,000	2	cc. 3 injections	50	Died 9 hours				0
28	37	1:200,000	1	cc.	10	Died 66				0
23	37	1:200,000	1	cc.	10	Died 68				0
34	33	1:200,000	2	cc.	10	Died 69				0
30	33	1:200,000	2	cc. 2 injections	20	Survived	18	20 mgm.	Died 25	1
36	33	1:200,000	2	cc. 2 injections	10	Survived	18	10 mgm.	Died 134	2
62	32	1:100,000	2	cc. 3 injections	10	Survived	3	3 M.L.D. gm. diphtheria toxin	Died 2 days	0
56	32	1:100,000	2	cc. 3 injections	10	Survived	3	3 M.L.D. gm. diphtheria toxin	Died 2 days	0

killed in 17 and 26 minutes by the injection of 7 mgm. of histamine per 100 grams. No accessory tissue was found at autopsy. Following the injection of 1 or 2 cc. of adrenalin chloride, 1:100,000, (2 or 3 injections 3 hours apart in 6 cases) and 10 or 20 mgm. of histamine per 100 grams, four rats survived, one died in 5 hours and 38 minutes, and one died in about 9 hours. Two of the survivors were killed two days later by similar doses of histamine. One of these and the one which died 9 hours after the injection of adrenalin and histamine were found at autopsy to have gross accessory cortical tissue. Three days later the other two survivors were given injections of diphtheria toxin, 3 M.L.D. per gram of body weight. Both died in two days. No gross accessory tissue was found at autopsy. Following the injection of adrenalin, 1:100,000, and 30, 40 and 50 mgm. of histamine per 100 grams, only one rat survived for a longer period than might be expected. This was one which had received 50 mgm. per 100 grams and which died in about 9 hours after the injection. Following the injection of 2 cc. of adrenalin, 1:200,000 (2 injections 3 hours apart) and 10 or 20 mgm. of histamine per 100 grams, two rats survived. Both were killed eighteen days later by similar doses of histamine without the previous injection of adrenalin. Both were found to have gross accessory cortical tissue.

Of twenty-two suprarenalectomized rats which received injections of 1 or 2 cc. of various dilutions of adrenalin chloride solution and 10 or 20 mgm. of histamine per 100 grams, twelve survived and four died after a survival period which was much longer than any observed following similar doses of histamine without the previous injection of adrenalin. That the survival was not due to the presence of accessory cortical tissue was shown by the autopsy findings and the results with subsequent injections of diphtheria toxin. That the doses of histamine used were truly lethal for suprarenalectomized rats was shown by the fact that the same rats were killed later by similar or smaller doses of histamine without previous injection of adrenalin. The presence or absence of gross accessory cortical tissue did not alter the results. It is concluded that in suprarenalectomized rats intraperitoneal injections of adrenalin chloride solution afford partial or complete protection in the majority of cases from the fatal effects of lethal doses of histamine acid phosphate.

These results, together with the results reported above, may be taken as evidence that the increased susceptibility to histamine in suprarenalectomized rats is due to medullary rather than to cortical defect. An obvious criticism of such a conclusion would be that it is not probable that a rat could normally secrete as much adrenalin as was used to protect the animals in the experiments described here. Moreover, it was not possible to protect the rats consistently from very large doses not fatal in normal rats. It must be remembered, however, that the intraperitoneal injection

of adrenalin chloride solution is a far different thing from the secretion of adrenalin into the blood from the suprarenal medulla. It is known that adrenalin is rapidly destroyed in the blood and tissues. It is probable that only a fraction of the injected adrenalin actually reached the blood stream without undergoing chemical change. It is also probable that what did reach the circulation could have only a fleeting action before it was destroyed, and could very imperfectly imitate the normal secretion from the glands. The fact that some measure of protection was afforded by this very imperfect imitation of the normal secretion of adrenalin from the intact medulla is considered to be significant evidence for the conclusion already stated.

Banting and Gairns (1926) reported that they were not able to protect a suprarenalectomized dog from the fatal effects of a small dose of histamine by means of subcutaneous injections of adrenalin. Only one case was reported in their paper, and furthermore I was not able to protect suprarenalectomized rats by means of subcutaneous injections of adrenalin. Intraperitoneal injections, however, were effective. Undoubtedly adrenalin is absorbed much more quickly from the peritoneal cavity than from the subcutaneous tissues. Moreover, the quicker the adrenalin can get into the circulation the more chance it has of antagonizing the effect of histamine before it is destroyed. The dilating action of histamine on the blood vessels is known to be very rapid in its inception.

Discussion. Dale and Richards (1918) and Burn and Dale (1926) have accumulated much evidence to support their theory that there is a special physiological antagonism between adrenalin and a capillary-dilator principle, which probably is histamine. Dale and Richards suggested that both of these substances act on the capillaries and that the liberation of proper amounts of each would provide a perfect fine adjustment of capillary tone. Burn and Dale stated that in the perfused organ of the cat adrenalin has "a unique effectiveness in reviving the tone of the vessels dilating with histamine." Kellaway and Cowell (1922) showed that in the cat the injection of small doses of histamine causes increased output of adrenalin as shown by the paradoxical pupil dilatation. They suggested that a test based on this fact could be used as a "guide to the amount of functioning medulla in animals with varying degrees of adrenal insufficiency." From a series of experiments with cats in which the suprarenals had been removed or the medulla alone destroyed, and with others in which the cortex alone had been damaged, they concluded that "the concentration of the blood in animals dying of adrenal insufficiency is due to cortical defect, ... the altered concentration reaction to small doses of histamine in such animals is due to medullary defect," and that "both cortical and medullary defect appear to play a part in the 'collapse' type of response to the injection of small doses of histamine." They showed, furthermore, that the sub-

cutaneous injection of adrenalin antagonizes the action of histamine in causing concentration of the blood and masks the hyper-sensitiveness to histamine in animals with suprarenal insufficiency. They concluded that, in normal animals, the increased output of adrenalin which histamine causes protects the animal against histamine collapse. The results reported in this paper tend to confirm the conclusions of Kellaway and Cowell. In fact, it is my opinion that the increased susceptibility to histamine in rats with suprarenal insufficiency is largely due to lack of medullary tissue. Certainly the presence of cortical tissue alone, in amounts comparable to the normal and functional as far as can be determined by a number of tests, does not protect against histamine. On the other hand, the presence of a small amount of chromaffin tissue does appear to protect against histamine. Furthermore, the administration of adrenalin in small amounts and in a way which must be far different from the constant secretion from the normal glands does protect, to a certain measure, rats with no cortical tissue at all. It follows that increased susceptibility to histamine is not a test for cortical insufficiency. Rather, it might be used as a test for the absence of functional medullary tissue.

The results reported here are significant in the light of a question which has recently been reopened by the work of Dragstedt, Wightman and Huffman (1928). They showed that the minimal effective dose of adrenalin on sustained administration in the unanesthetized dog produces a sustained pressor effect. Moreover, the minimal effective dose was found to be comparable to the figure obtained by other investigators for the average normal output of adrenalin in the dog. This work does away with the chief objections to the tonus theory of medulliadrenal function; namely, that in anesthetized animals adrenalin is not normally secreted in amounts necessary to produce sustained pressor effects, and that the minimal effective dose produces a depressor effect. Their results are compatible with the theory of Dale and others that adrenalin is a factor in the maintenance of normal capillary tone. The results reported here, taken in conjunction with the theory of a physiological antagonism between adrenalin and histamine, should be considered in connection with the rôle of the adrenals in secondary shock, with the rôle of histamine in shock, and with the use of adrenalin in the treatment of shock.

SUMMARY

1. The lethal intraperitoneal dose of histamine acid phosphate for normal albino rats is more than 100 mgm. per 100 grams of body weight.

2. Single suprarenalectomy or a blank operation does not increase the susceptibility of the rat to histamine.

3. The minimal lethal dose of histamine for doubly suprarenalectomized

rats was found to be about 7 mgm. per 100 grams. The survival period following this dose is usually one hour or less.

4. The increased susceptibility to histamine after double suprarenalectomy is constant, at least up to five months after operation.

5. Suprarenalectomized rats possessing gross accessory cortical tissue or autoplastic cortical transplants, in amounts comparable to the normal and functional as far as can be determined by the condition of the rats, the fertility of females and tests with diphtheria toxin, are as susceptible to histamine as rats having no cortical tissue and suffering from cortical insufficiency.

6. The presence of accessory chromaffin tissue appears to protect suprarenalectomized rats from the fatal effects of doses of histamine which are lethal for rats having no such tissue.

7. Intraperitoneal injections of small amounts of adrenalin chloride solution afford partial or complete protection from the fatal effects of lethal doses of histamine.

8. It is concluded that the increased susceptibility to histamine of suprarenalectomized rats is largely due to the lack of medullary tissue, rather than to the lack of cortical tissue.

9. It follows that increased susceptibility to histamine is not a test for cortical insufficiency. It might be used as a test for the absence of functional medullary tissue.

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STUDIES ON SUPRARENAL INSUFFICIENCY

III. The Effect of Thyroidectomy and of Forcing Fluid on Suprarenal Insufficiency in Rats

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Much has been written concerning the theory that the suprarenal glands play an important part in the protection of the body against infection and intoxication. In recent years many studies have been published on the increased susceptibility of suprarenalectomized animals to intoxication of various kinds. The literature is well reviewed and discussed in the papers of Lewis (1923) and Scott (1923, 1924) and in the more recent papers of Jaffe (1927), and of Torino and Lewis (1927). In the case of certain toxins it appears quite definitely that the increase in susceptibility after suprarenalectomy is due to the loss of the cortical tissue (Scott, 1924; Jaffe, 1926). It is probable that the same will be proven in the case of increased susceptibility to many other of the poisons which have been investigated; although in the case of one, namely, histamine acid phosphate, the loss of the secretion of the medullary tissue seems to be largely responsible for the increase in susceptibility (Wyman, 1928a).

The mechanism whereby the suprarenal cortex protects or aids in protecting the organism from intoxication is unknown. We do not know whether the protection comes directly from a hormone secreted by the cortex or secondarily through the maintenance of some other protective mechanism by an influence from the cortex. In fact, we are not sure that the cortex actually produces a hormone, although the recent discovery of Hartman, MacArthur and Hartman, (1927) points to such a possibility. It has been suggested that the protective function of the suprarenals is not of the nature of a detoxication as indicated by the earlier hypothesis, but that it is due to the maintenance of some normal metabolic reserve factor (Scott, 1924). Belding and Wyman (1926) showed that suprarenalectomized rats, four hours after receiving an intraperitoneal injection of diphtheria toxin equivalent to 1 M.L.D. per gram of body weight, have 2.75 times as much toxin in the blood as normal rats. They suggested that suprarenal insufficiency renders less effective the normal mechanism

of the rat for the elimination or destruction of diphtheria toxin. In making this suggestion the authors did not have in mind an actual detoxication by a suprarenal product. What was meant was a disturbance, possibly metabolic in nature, of some other part of the body which normally has to do with non-specific resistance to intoxication.

Zwemer (1927), Swingle (1927) and Swingle and Eisenman (1927) found concentration of the blood, diminished urine output, and marked symptoms of dehydration in suprarenalectomized cats. Swingle suggested that the death of suprarenalectomized cats may be due to hypoglycemia or to acid intoxication. He attributed the acidosis to kidney failure although he later concluded that the acidosis is extrarenal in origin and only partially dependent upon kidney injury (Swingle, Wenner and Stanley, 1928). Zwemer found that the oral administration of sufficient quantities of a 5 per cent glucose solution will prolong the life of suprarenalectomized cats. He also found that removal of the thyroid glands of suprarenalectomized cats prolongs their life, whereas feeding thyroid substance hastens death. He regards the thyroid as a dehydrating agent and since the symptoms of suprarenal insufficiency in cats resemble those of anhydremia, he explains his results on the basis of decreased elimination of water in the one case and of increased elimination of water and added dehydration in the other. Corey (1927) found that forcing large quantities of fluid, especially solutions of NaCl and of glucose, by the oral route prolongs the life of suprarenalectomized cats. He suggested that this procedure relieves the dehydration and by the consequent diuresis flushes out toxic substances.

In the light of the experiments reported by Swingle, by Zwemer, and by Corey, two interpretations may be put upon the results of Belding and Wyman. One is that the larger amount of toxin remaining in a given amount of the blood of suprarenalectomized rats is due to blood concentration and not due to a larger amount of toxin remaining in the whole organism. The other is that because of dehydration and impaired kidney function less toxin is eliminated than in normal rats as was suggested by the authors. Following is a report of an attempt to discover whether or not relief of dehydration by methods which have been successful in prolonging the life of suprarenalectomized cats can alter the effects of suprarenal insufficiency in rats.

METHODS. A mixed stock of colored varieties of the albino rat was used. The care of the animal colony, diet, and the technique of suprarenalectomy and of control operations have been described in previous papers (Belding and Wyman, 1926; Wyman, 1928b). Thyroidectomy was performed with aseptic technique under ether anesthesia. It was found that it is necessary to ligate only the superior thyroid arteries. Little or no bleeding occurs from the inferior poles if the arteries are pinched with forceps before

cutting. Care must be taken not to injure the recurrent laryngeal nerves which are closely attached to the thyroid along the mesial border of each lobe. These nerves were injured in nine animals, causing difficulty in respiration and death on the same or the next day. In these animals the lungs were found at autopsy to be profoundly congested. Care must be taken to remove all of the thyroid tissue which lies between the trachea and esophagus, firmly attached to the former. Blunt dissection is the most satisfactory method of removing the gland. The parathyroids were dissected out in each case and left in place, usually with a stalk of tissue connecting the gland to the surrounding tissues. When this could not be done the parathyroid was dissected free from the thyroid and replaced. No clinical symptoms of parathyroid insufficiency were seen in any of the rats during the time that they were allowed to live.

Diphtheria toxin, lot no. 46(3) (M.L.D., 0.002 cc.) obtained from the Massachusetts Department of Public Health, was given by intraperitoneal injection, the dose being measured in M.L.D. per gram of body weight of the rat. Ergamine acid phosphate (Burroughs Wellcome & Co.) was given by intraperitoneal injection in a solution containing 50 mgm. of ergamine per cubic centimeter of sterile distilled water. The doses are reported as milligrams of ergamine acid phosphate per 100 grams of body weight. A sterile, 5 per cent solution of glucose (C.P. anhydrous, Special Chemicals Co.) in distilled water was injected intraperitoneally. By using sufficient care intraperitoneal injections may be made without causing the rats undue alarm or discomfort.

Thyroidectomy and double suprarenalectomy. Thirty adult rats survived thyroidectomy and appeared to be in good condition, resembling normal or control animals in every way except for a change in weight. Both suprarenal glands were removed from these animals by the dorsal route from 7 to 14 days after thyroidectomy. Seventeen had gained weight and thirteen had lost weight during the period between the two operations. The gains were from 1 to 83 grams averaging 23.6 grams, and the losses were from 1 to 30 grams averaging 17.6 grams. Of these rats 44.4 per cent died of acute or subacute suprarenal insufficiency from 7 to 21 days after suprarenalectomy, the majority of deaths occurring between 15 and 20 days after operation. These animals showed the usual clinical symptoms of suprarenal insufficiency which are characteristic in rats, and about the same number as in a series of rats suprarenalectomized without previous thyroidectomy exhibited the characteristic autopsy findings, including asthenia, diarrhea, congested alimentary canal, bloody intestinal contents, blood around the eyes and nose, congested lungs, congested or mottled kidneys, congestion or hemorrhagic spots in the thymus, and enlarged thymus and lymph glands.

Of 247 rats, which were doubly suprarenalectomized without previous

treatment during the same time that the experiments on the effect of thyroidectomy were being done and which were from the same stock and were kept under the same conditions, 117 or 47.4 per cent died of acute or subacute suprarenal insufficiency during the first month after operation. In calculating the percentage of mortality due to suprarenal insufficiency all rats showing at autopsy evidence of respiratory infections or other diseases which might contribute to the cause of death were excluded from all series under consideration. It was noticed that rats having severe respiratory infections almost invariably died within a week or two after suprarenalectomy whereas they usually lived indefinitely if left alone.

The autopsy findings in rats dead of acute suprarenal insufficiency have been accurately described by Jaffe and others. Scott (1927) called attention to a latent abnormality in rats with suprarenal insufficiency, which manifests itself by mucosal hemorrhages when the insufficiency becomes lethal. Mucosal hemorrhages were striking findings in many of the rats in the series described above. In many cases the intestinal contents were blood-stained or were actually large amounts of little else but blood. The hemorrhage appeared to be confined to the ileum in practically all cases. Blood oozing from the mucous membranes around the eyes or from the nose was frequently seen. Bloody eyes were often noticed in an animal suffering from acute insufficiency. Although the pathology of suprarenal insufficiency in rats has been well described by others it might be worth while to give the percentages of the most characteristic autopsy findings in a series of 174 rats dead of acute or subacute insufficiency. They are as follows; diarrhea 54 per cent, congested stomach 59.8 per cent, congested intestines 74.7 per cent, blood in intestinal contents (ileum) 27.6 per cent, congested lungs 55.7 per cent, bloody eyes 27.6 per cent, bloody nose 8.6 per cent, congested kidneys 24.1 per cent, congestion or hemorrhagic spots in thymus 15.5 per cent, enlarged thymus 20.7 per cent.

The similarity of symptoms and autopsy findings and the similar acute and subacute mortality percentages (44.4 and 47.4 per cent) in the thyroidectomized-suprarenalectomized and in the suprarenalectomized series indicates that previous thyroidectomy does not mitigate acute or subacute suprarenal insufficiency in rats. Of the thyroidectomized-suprarenalectomized rats which survived for further experimentation 10 gained weight in the period between the two operations, but 10 lost weight (from 16 to 58 grams each) and only three gained (from 8 to 22 grams each) during the first three weeks following suprarenalectomy. Of those that gained, one was found at autopsy to possess gross accessory cortical tissue. This further indicates that thyroidectomy does not have a beneficial effect upon succeeding suprarenal insufficiency.

Intraperitoneal injections of various amounts of diphtheria toxin were given to 14 normal or blank operated rats, to 19 suprarenalectomized rats

from three weeks to two months after operation, and to 10 thyroidectomized-suprarenalectomized rats about three weeks after suprarenalectomy. The lethal dose for normal or blank operated rats averaged 6 M.L.D. per gram of body weight. The lethal dose both for the suprarenalectomized rats and for the thyroidectomized-suprarenalectomized rats averaged 2.75 M.L.D. per gram. Both sets of suprarenalectomized rats were, therefore, 2.18 times as susceptible to diphtheria toxin as the controls. This agrees fairly well with the figure of 2.5 for suprarenalectomized rats found by Belding and Wyman (1926). The autopsy findings in both sets of suprarenalectomized rats killed by diphtheria toxin were precisely similar, a constant finding being an edematous hemorrhagic condition of the lungs. Evidently thyroidectomy previous to suprarenalectomy does not influence the increased susceptibility to diphtheria toxin.

The minimal lethal dose of histamine acid phosphate which could be depended on to kill a suprarenalectomized rat was found to be 7 mgm. per 100 grams of body weight although 5 mgm. per 100 grams usually kills. Normal rats survive up to 100 mgm. per 100 grams (Wyman, 1928a). This increase in susceptibility to histamine has been demonstrated by a number of investigators. Five thyroidectomized-suprarenalectomized rats were killed quite promptly by doses of 7 mgm. per 100 grams in 3 cases and by doses of 5 mgm. per 100 grams in 2 cases. Evidently thyroidectomy does not influence the increased susceptibility to histamine poisoning in suprarenalectomized rats.

It has long been known that it is extremely difficult to be sure of removing all traces of thyroid tissue from the rat since the gland is closely adherent to the trachea, and that small pieces which may remain are likely to regenerate. Serial sections of the larynx and trachea with the surrounding tissues in the field of operation were examined in the case of 18 of the rats studied. In 12 cases small bits of thyroid tissue were found in the region around the parathyroids or near the points where the superior thyroid arteries were ligated. Sometimes two masses were found, one on each side. In other cases a single mass was found on one side only. In no case were more than two such masses found. They varied in size from a few follicles (3 to 6) to a mass half the size of the parathyroid. In the other 6 cases no thyroid tissue was found. The distribution of these 18 cases was as follows; of rats dead from suprarenal insufficiency, 3 with and 2 without thyroid tissue; of rats killed with diphtheria toxin, 5 with and 3 without thyroid tissue; of rats killed with histamine, 4 with and 1 without thyroid tissue. Since these masses were so small, as compared with a normal gland, it is probable that the animals possessing them were decidedly deficient as far as thyroid secretion was concerned. Furthermore, the similarity of experimental results in rats with such thyroid

fragments and in those having no identifiable thyroid tissue makes it seem reasonable to treat both groups alike in drawing conclusions.

Forcing fluid. Cunningham (1920) found that in rats intraperitoneal injections of 10 cc. of a 10 per cent solution of dextrose were completely absorbed in about 12 hours. Daily injections of this amount of fluid produced no ill effects. He later demonstrated that the absorption of 5 per cent glucose solution from the peritoneal cavity takes place through the blood vascular system (Cunningham, 1927). Hence, the intraperitoneal injection of a 5 per cent solution of glucose appeared to be a good method of forcing fluid in rats.

Various amounts of a sterile 5 per cent solution of glucose, warmed to body temperature, were slowly injected intraperitoneally into 40 doubly suprarenalectomized rats. The first injection was given from two weeks to one month after operation. Of these 40 rats 25 died after the injection of glucose solution. Three died from 5 to 12 hours after a single injection of 15 or 20 cc. Nine died within 24 hours after a single injection of 10 cc., and four died after an injection of 6 cc. Two died within 24 hours after the last of 2 daily injections of 6, 8 or 10 cc., five died within 24 hours after the last of 3 such daily injections, and two died after 4 such injections. In most cases a few cubic centimeters of fluid were found remaining in the peritoneal cavity at autopsy. This fluid was clear in all cases, being colorless in some and tinged with red in others. Autopsy findings characteristic in death from suprarenal insufficiency were found in about half of these rats. Evidently suprarenalectomized rats are susceptible to the intraperitoneal injection of these amounts of glucose solution. The factors responsible for death were not determined.

The rats which survived the injections of glucose solution were given injections of histamine or of diphtheria toxin. Ten rats which had received 2, 3, 4 or 5 daily injections of 10 cc. of a 5 per cent solution of glucose were given intraperitoneal injections of various amounts of a solution of histamine from 5 to 24 hours after the last injection of glucose solution. One rat survived a dose of 15 mgm. per 100 grams. This rat was found at autopsy to have a large mass of accessory suprarenal tissue, probably containing accessory medullary tissue. The other nine were killed by doses of 5 mgm. per 100 grams in 3 cases, 7 mgm. per 100 grams in one case, and 10 mgm. per 100 grams in 5 cases. Evidently the administration of glucose solution did not influence the increased susceptibility to histamine poisoning of suprarenalectomized rats.

Four rats were given injections of glucose solution followed by injections of diphtheria toxin. One received six daily injections of 10 cc. of glucose solution and an injection of 3.5 M.L.D. per gram of diphtheria toxin five hours after the fifth injection of glucose. The first injection of glucose was given 25 days after double suprarenalectomy. This rat died

3 days after the injection of diphtheria toxin. The other three received seven daily injections of 8 cc. of glucose solution and an injection of 3 M.L.D. per gram of diphtheria toxin five hours after the fifth injection of glucose. The first injections of glucose were given 17 or 18 days after suprarenalectomy. These rats survived. Ten or eleven days after the first dose of diphtheria toxin they were given another injection of 3 M.L.D. per gram without previous treatment with glucose solution. They survived this second injection. A week and a half later they were killed for autopsy and each was found to have a large mass of accessory cortical tissue in the suprarenal sites. This tissue was sectioned and identified microscopically. The work was interrupted at this point. Although the one case is not sufficient evidence for absolute conclusions it is suggestive. It seems likely that the administration of glucose solution does not influence the increased susceptibility to diphtheria toxin of suprarenalectomized rats. The other three cases are interesting in that they are confirmatory of the idea that increased susceptibility to diphtheria toxin after suprarenalectomy in rats is due to loss of cortical tissue. It has frequently been my experience that suprarenalectomized rats possessing gross accessory cortical tissue are not more susceptible than normal rats to diphtheria toxin.

Discussion. We have no proof that dehydration or blood concentration are factors in suprarenal insufficiency in rats. It is quite possible that the changes in blood chemistry and the constitution of body fluids which are found in the acute type of suprarenal insufficiency of cats and dogs are not characteristic of the subacute or chronic types of insufficiency of rats. Studies on these points are in progress in this laboratory. Certainly thyroidectomy does not have the beneficial effect upon suprarenal insufficiency in rats that it has been demonstrated by Zwemer to have in the case of cats. Furthermore, forcing glucose solution by intraperitoneal injection in the quantities used in the work reported here is far from being beneficial. To the contrary it appears to be distinctly harmful. Whether the harmful effects are due to the presence of the solution in the animal or to the trauma involved in introducing such quantities into the peritoneal cavity was not determined. Injections of similar amounts of fluid do not appear to be injurious to normal rats.

Scott (1924) could not protect suprarenalectomized rats from the fatal effects of bacterial intoxication by means of glucose given intravenously or intraperitoneally. Torino and Lewis (1927) could not increase the tolerance for morphin of normal rats by means of repeated glucose injections. These investigators, however, were attempting to control hypoglycemia without reference to the fluid content of the body. The failure of thyroidectomy and of forcing glucose solution to reduce the increased susceptibility to toxins in suprarenalectomized rats indicates that this suscepti-

bility is a thing apart from the factors responsible for death following total removal of cortical tissue. It is possible that blood changes similar to those seen in cats arise in rats when suprarenal insufficiency becomes acute immediately preceding death. Increased susceptibility to poisons, however, is characteristic in subacute and chronic insufficiency in rats and is not limited to a period attended by more severe symptoms, such as the period just before the death of the animal. This suggests that impaired ability to flush toxins out of the body because of dehydration is not an important part of the picture of increased susceptibility. In the case of diphtheria toxin the normal mechanism of destruction or elimination may not be connected with the fluid content of the body. Further light on the validity of the conclusion of Belding and Wyman may be forthcoming in a study of the blood of suprarenalectomized rats.

SUMMARY

1. Thyroidectomy previous to suprarenalectomy in rats does not alter the symptoms or the mortality percentage of acute and subacute suprarenal insufficiency.

2. Both suprarenalectomized and thyroidectomized-suprarenalectomized rats are between two and three times as susceptible to diphtheria toxin as normal or blank-operated rats. Thyroidectomy previous to suprarenalectomy in rats does not influence the increased susceptibility to diphtheria toxin.

3. Thyroidectomy does not influence the increased susceptibility to histamine poisoning in suprarenalectomized rats.

4. Intraperitoneal injections of from 6 to 10 cc. of a 5 per cent solution of glucose may cause the death of suprarenalectomized rats. Normal rats do not show any ill effects after such injections.

5. Daily injections of 10 cc. of a 5 per cent solution of glucose do not influence the increased susceptibility to histamine poisoning in suprarenalectomized rats.

6. In one case it was demonstrated that the intraperitoneal administration of glucose solution apparently does not influence the increased susceptibility to diphtheria toxin in suprarenalectomized rats.

7. It is suggested that in rats dehydration is not an important factor in subacute and chronic suprarenal insufficiency and the attendant increased susceptibility to intoxication.

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THE RELATION OF THE BLOOD SPECIFIC GRAVITY TO THE CELL COUNT, HEMOGLOBIN LEVEL, CELL VOLUME AND TOTAL BLOOD VOLUME IN PIGEONS

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During the course of a study of the anemia of pigeons suffering from rice disease (Barlow and Biskind, 1928), a wide range of variation in the specific gravity of the whole blood was noted (1.040 to 1.071) according to the nutritional condition of the birds. However, a rather wide range of variation (1.054 to 1.071) was also noted in normal pigeons under standard laboratory conditions. As the variations in specific gravity did not always appear to harmonize satisfactorily with the cell counts, either of normal birds or of those suffering from rice disease, a further study seemed advisable including also readings of the hemoglobin level, cell volume and total blood volume.

METHODS. The plan of procedure was to compare the red cell counts, hemoglobin percentages, cell volumes and total blood volumes of a rather large series of birds (both normal and anemic) with the corresponding whole blood specific gravities in order to learn the relationship and the cause of the variations of these observations. In a number of experiments the specific gravity of the serum was also noted. The cell count was determined with a standardized Leitz chamber; hemoglobin by the Newcomer method; whole blood specific gravity by means of a mixture of chloroform and benzene, and cell volume by the Van Allen hematocrit. These readings were taken at three day intervals until three to four observations were obtained. The pigeon was then decapitated and the blood obtained by hemorrhage (usually 60 to 65 per cent of the total blood volume), was defibrinated and weighed. In representative experiments the total blood volume was determined directly by the modified Welcker method of Barlow and Biskind. The specific gravity of the defibrinated blood and serum was also determined gravimetrically. The cell volumes in the control series were determined by three methods; that of the whole blood by the hematocrit, while that of the defibrinated blood was esti-

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mated also colorimetrically by the method of Hoppe-Seyler, and further by measuring the electrical conductivity of the whole defibrinated blood and serum by the procedure described by Stewart (1899). In the latter method the resistances, in ohms, of the whole blood and serum to the passage of a current between two platinum electrodes were utilized for determining the proportion of cell to serum by the formula,

$$P = \frac{K\left(\frac{1}{R}\right) - \left(\frac{1}{R}\right)^2}{\frac{1}{R_2}}$$

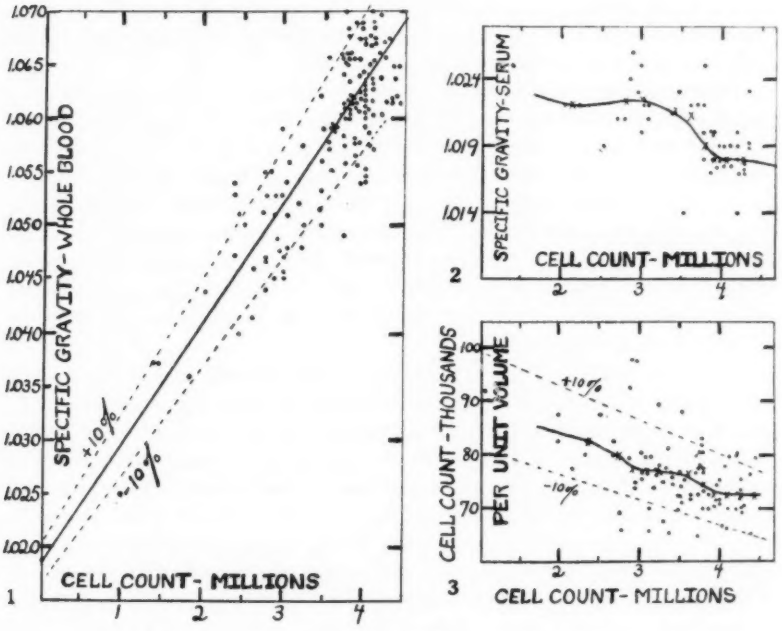
in which P equals the per cent or cubic centimeters of serum in 100 cc. of whole blood, K is a constant and equals 174 for dogs' blood (for pigeons' blood the factor 158 gives results that agree better with those of the colorimetric control method); R equals the resistance in ohms of whole blood (defibrinated) and R_2 the resistance in ohms of the serum.

Comparison of the results of the three cell volume methods. On nine pigeons the blood cell volumes were determined in triplicate for the purpose of comparing the data by the three methods. The colorimetric determinations may be used as standard since they should theoretically be the most exact. The colorimetric corpuscle volume ranged from 40.2 to 57.5 per cent with a median of 51.6. The hematocrit readings agreed closely but were almost uniformly a trifle high with a median of 52.9 per cent instead of 51.6 (colorimetric). This is a median difference of +1.3 per cent and in five of the nine birds the range lies between 1.3 and 1.5 per cent. The cell volumes of 68 normal pigeons examined by the hematocrit vary from 48.1 to 61.1 per cent with a median value of 54.8. The cell volumes determined by the conductivity method with K equal to 158, were generally somewhat lower (median −1 per cent) than the standard readings but scatter rather widely with a range of −10.5 to +6.5 per cent on either side of them. Five-ninths of the conductivity readings, however, lie between −3.9 and +2.8 per cent of the colorimetric observations. The occasional wide departures from the controls render the conductivity method unsatisfactory in our hands, although we are ready to concede that they were probably due to errors of technic which we were unable to avoid. On the other hand the hematocrit method, which is the most convenient of those tested, gives uniformly satisfactory results.

Comparison of the Hammerschlag and gravimetric specific gravity methods. The specific gravity of the whole blood, determined by Hammerschlag's method does not differ significantly from corresponding data obtained gravimetrically from the same blood after defibrination. The range by the former was from 1.054 to 1.068 with a median of 1.0615 for the series of 30 birds. Gravimetrically, the range was 1.055 to 1.071 with a median

of 1.062. Since the data by the two methods were quite uniform, all specific gravity figures of the whole blood used in the remainder of the text, refer to data obtained by the Hammerschlag procedure.

Relation of the whole blood specific gravity to the cell count. In order satisfactorily to show the relationship existing between these several data, it was necessary to use a rather large series which includes 68 normal pigeons with red cell counts ranging from 3,780,000 to 4,520,000 and 42 anemic birds suffering either from rice disease or starvation with cell counts ranging between 1,000,000 and 3,440,000.



Figs. 1 to 3

The specific gravity of the whole blood of pigeons may vary from 1.028 to 1.075 with a range of 1,000,000 to 4,520,000 red cells per cubic millimeter of blood according to the nutritional state of the birds. In figure 1 the individual cell counts have been plotted against the corresponding whole blood specific gravities. The solid line drawn between 1.01875, the median specific gravity of the serum and 1.064, the median specific gravity of the whole blood of birds with normal counts, represents the theoretical relationship existing between such data, if the specific gravity and cell count vary directly. The broken lines are drawn 10 per

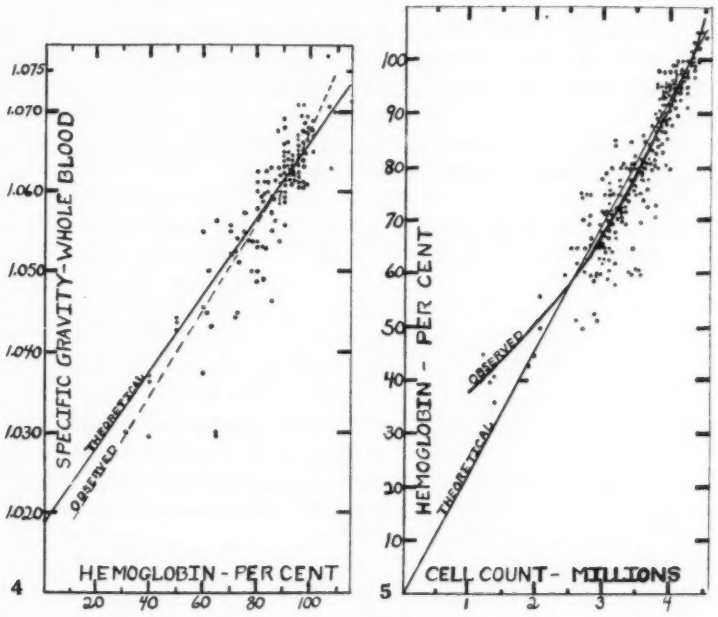
cent on either side of this theoretical value. It may be noted that the dots, representing the actual observations, are distributed symmetrically about the theoretical median and agree with it so closely that nearly all fall within the ±10 per cent zone. It may therefore be concluded that the specific gravity of the whole blood of pigeons is essentially a function of the cell count, although it may be modified to a limited extent by other factors, such as the specific gravity of the serum. The latter however is not of great importance, for the correspondence is not materially improved if the individual data in figure 1 are corrected for this factor. It is conceivable that these divergences might be due to differences in the average size of the corpuscles, but this was not the case, as the number of cells per unit cell volume may be greater than normal with data on either side of the theoretical median.

Relation of the serum specific gravity to the cell count. The serum specific gravity of pigeon blood may vary from 1.025 with a cell count of 1,760,000 to 1.017 with a cell count of 4,340,000. Figure 2 shows that as the cell count decreases, especially below 3,500,000, the median specific gravity of the serum tends to rise, although the individual data are scattered. We are not prepared to offer an explanation of this apparent relation between the anemia and the serum specific gravity, although it is conceivable that it may be connected with changes in the imbibing power of the serum colloids such as were noted by Thomas and Andrews (1928) in cases of human edema.

Relation of the red cell count to cell size. The cell size or volume of adult normal pigeons, as indicated by the cell count per unit cell volume (fig. 3), varies as a rule within a range of ±5 per cent of the median for the series. A few birds however deviate by 10 per cent or more from the mean. The travelling median for the entire series has a definite slant, so that the cell volume decreases with the cell count; or in other words, the cell size in nutritional anemia tends to decrease as the anemia develops. There is also a much greater divergence of the anemic animals from the mean, i.e. a large number of the anemic pigeons have either an excessive proportion of small corpuscles or show a reduction in the number of cells proportional to the degree of inanition. On examining these data, it was found that the former group, i.e., those having a large proportion of small corpuscles, was suffering from chronic inanition due to prolonged polished rice feeding. These animals show a true secondary anemia. The group having a low cell count of relatively large corpuscles, on the other hand, comprises birds on a total fast. The relatively short survival period (9 to 12 days) of fasting birds is usually insufficient, in point of time, for the development of a secondary anemia and in a certain percentage of cases the cell count reduction without any apparent change in the cell size is due to blood dilution, *i.e.*, the development of hydremic plethora. The explana-

tion of the slant of the median which develops coincident with the anemia is not clear.

Relation of the whole blood specific gravity to the hemoglobin. If the hemoglobin content of the blood were strictly proportional to the corpuscle count, the specific gravity of the whole blood would vary with the hemoglobin percentage since the whole blood specific gravity corresponds rather closely with the red cell count. The theoretical relation between the specific gravity and hemoglobin per cent is shown by the diagonal line drawn between the median specific gravity of the serum and the inter-



Figs. 4 and 5

section of the median hemoglobin and whole blood specific gravity values in figure 4. The broken line represents the travelling median of the actual data which are indicated as dots. It will be noted that the observed median is more steep than the theoretical. With hemoglobin values above 95 per cent, the data are from 1 to 10 per cent (median +3.7) above the theoretical. With values between 85 and 95 per cent, the correspondence of the two medians is exact. Below 85 per cent however the data are more scattered, i.e., deviate more widely from the mean, and are below the theoretical median. The differences between the theoretical and ob-

served medians are due primarily to corresponding divergences of the hemoglobin from the cell count but are also associated with the alteration in the cell size as discussed in relation to figure 3.

Correlation of the red cell count with the hemoglobin per cent. In figure 5 the individual red cell counts (abscissa) have been plotted against the corresponding hemoglobin percentages (ordinate). The lighter solid line represents the theoretical relation which should exist between these observations if the hemoglobin and cell changes are directly proportional. The heavy solid (curved) line shows the travelling median for the observed data. It may be seen that these are distributed in fairly close proximity to the theoretical but that the direction of the median is a logarithmic curve instead of a straight line. The hemoglobin or red cell values for the particular set of conditions represented may thus be determined by the following formula, which was derived by prolonging the logarithmic curve of the hemoglobin values, y, to a point intersecting the prolongation of the abscissa, which represents the red count (fig. 5), and using the general equation for a straight line;

$$\text{Log } Y = X \frac{\text{Log } Y}{X + A} + \text{Log } B$$

resolving

$$1 - \frac{\text{Log } B}{\text{Log } Y} = \frac{X}{X + A}$$

in which A is a constant equal to 1.2505, B is a constant equal to 1.491 and Y equals the log of the hemoglobin per cent, and X equals the cell count.

The hemoglobin thus tends to increase as a geometric power of the blood count; or in other words, as the number of corpuscles increase, their richness in hemoglobin or color index also rises.

SUMMARY AND CONCLUSIONS

Using the colorimetric procedure as a standard, the normal cell volume of pigeon blood is of the order of 51.6 per cent. The readings obtained by the conductivity method were more variable, in our hands, than the other two methods tested and gave results which varied from −3.9 to +2.8 per cent on either side of the standard observations. The hematocrit readings were in close agreement and were from 1.3 to 1.5 per cent higher than the parallel colorimetric determinations. The hematocrit therefore is reliable, quite satisfactory and the most convenient of the procedures tested.

The specific gravity of the whole blood of pigeons has a normal variation of from 1.054 to 1.070 with a median of 1.064. The specific gravity of the

whole blood determined by Hammerschlag's method does not differ significantly from gravimetric determinations of the same blood after defibrination.

When the whole blood specific gravity is plotted against the red cell count, the travelling median for the series corresponds closely with the theoretical relationship which should exist between these observations. Individual readings with similar counts may vary normally within a range of ± 10 per cent however.

The serum specific gravity of normal pigeon blood ranges from 1.016 to 1.020 with a median of 1.0187. As the nutritional anemia develops, the serum specific gravity scatters rather widely on either side of the travelling median for the series but in general increases from a normal of 1.0187 to 1.022. This change in the serum when used to correct the whole blood specific gravity does not significantly alter the relation existing between the latter and the red cell count.

The red cell size of pigeons varies within a range of ± 5 per cent of the median although individual readings may show a deviation of ± 10 per cent. As anemia develops, the cell size definitely increases and the deviation of the individual readings from the mean becomes greater, depending on the length of the period of development and the character of the anemia produced.

The relationship obtained by plotting the whole blood specific gravity against the hemoglobin per cent is also close, especially with hemoglobin values between 82 and 94 per cent. On either side of this range however, the deviation of the individual data increases and the observed median is somewhat steeper than the theoretical, due primarily to a change in the color index.

The values obtained by plotting the hemoglobin against the cell counts show that the hemoglobin increases as a geometric function of the cell count.

In correlating the rather wide variations observed in the whole blood specific gravity with either the serum specific gravity, cell count, hemoglobin percentage, cell volume or total blood volume, no absolutely quantitative relationship could be noted between the specific gravity and any of the other observations. However, the relation of the red cell count and the whole blood specific gravity is generally within 10 per cent of the theoretical for these observations.

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THE UTILIZATION OF ACETOACETIC ACID BY NORMAL AND DIABETIC DOGS BEFORE AND AFTER EVISCERATION

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Although derivatives chiefly of the fatty acids, the ketone bodies were early recognized to be intimately associated with carbohydrate metabolism, since they are excreted in abnormal amounts in diabetes, in fasted animals and, in man, when carbohydrate is excluded from the diet. The mechanism whereby glucose inhibits ketosis has received different explanations from time to time. Assuming that the appearance of the ketone bodies is associated with insufficient oxidation of carbohydrate Geelmuyden (1909), Woodyat (1910) and Ringer (1914) suggested that the antiketogenic action of carbohydrate in the animal body is due to a chemical reaction between glucose, or a closely related product, and one of the ketone bodies. Shaffer (1921) was led to a similar conclusion when he found that the addition of hydrogen peroxide to a strongly alkaline solution containing acetoacetic acid and glucose caused a rapid disappearance of the acetoacetic acid even at room temperature. In addition, this worker has been able to show that a certain ratio between ketogenic and antiketogenic substances must be maintained in the diet of human subjects in order to keep them free from ketosis.

While admitting the possibility of a chemical reaction in the animal organism between glucose and acetoacetic acid, a close examination of the assumptions upon which the calculations are based makes it difficult to accept the results obtained as valid proof of the view that the relationship of ketogenic to antiketogenic substances metabolized in the organism is the only factor determining the presence or absence of acetone bodies in the urine. Shaffer assumes that fat is used directly by the diabetic organism, whereas it has been shown by Chaikoff and Weber (1927a) and by others that a considerable proportion of the glucose excreted in the urine of the totally depancreatized fasted dog is derived from fatty acids. Moreover, the further assumption that is made in Shaffer's hypothesis that during the excretion of ketone bodies the organism is not oxidizing carbohydrate, has been definitely negatived by numerous observers, including Wierzuchowski (1925) and Deuel, Wilson and Milhorat (1927), who show that while the

phlorhizinized dog is excreting acetone bodies, it still retains the ability to oxidize carbohydrate.

In recently reported observations it was shown that glucose possesses no antiketogenic action in the completely depancreatized dog (Chaikoff and Weber, 1927b; Selle, 1927). On the other hand, the fact that the antiketogenic effect of glucose can be demonstrated in the fasted animal, in the phlorhizinized dog (Wierzuchowski, 1925; Deuel, Wilson and Milhorat, 1927) and in the fasted and diabetic patient, indicates that the antiketogenic action of glucose must act by stimulating the internal secretion of insulin. The view that glucose is a stimulus to the secretion of insulin seems well supported from the observations of Marks who found that the injection of glucose or adrenalin to rabbits whose livers had been rendered glycogen-free by previous feeding with thyroid, led to a rapid fall in the blood sugar and to death of the animals in hypoglycemic convulsions.

The present investigation was undertaken to determine the ability of the tissues of diabetic and normal animals to utilize acetoacetic acid, and to throw further light on the problem of ketogenesis and antiketogenesis.

PART I: EXPERIMENTS ON NORMAL AND EVISCERATED NORMAL DOGS.

Methods. Dogs after fasting from one to five days were anesthetized and the kidneys removed in order to prevent loss of the injected substances. The femoral veins of both legs were exposed, one being used for the injection of the acetoacetic acid, the other for withdrawal of blood for the analyses. After recovery, the animals received intravenously one gram of neutralized freshly prepared acetoacetic acid per kilo body weight, and the concentration of acetoacetic acid and of β-hydroxybutyric acid in the blood was determined at frequent intervals over a period of several hours. The results of dogs B and C are recorded in tables 1 and 2, while that of dog A is shown graphically in figure 1.

A similar study of the fate of acetoacetic acid was made in normal dogs upon which evisceration had been performed by Mann's technique. This consists, essentially, in subtotally ligating the vena cava a week or two previous to the performance of the evisceration, at which time the coeliac axis, superior and inferior mesenteric vessels are ligated, the vena cava and oesophagus clamped off below the diaphragm and this followed by excising of the liver and gastro-intestinal tract. The results of three such experiments are recorded in tables 3 (dog E) and 4 (dog F) and figure 2 (dog D).

In all cases autopsies were performed at the end of the experiments. No fluid was found in the peritoneal cavity in any of the experiments reported.

The terms normal and "eviscerated-normal" are used to distinguish the above two preparations from the diabetic and "eviscerated-diabetic," to which reference will be made later.

The acetone bodies of the blood were determined by the method of Van

Slyke and Fitz (1917). Muscle, after being frozen hard with liquid air and pulpified, was extracted for acetone bodies by the Shaffer-Marriott (1913) method, the final distillate obtained being treated as the filtrate in the Van Slyke method (1917). A somewhat similar method for determination of these substances in tissue has been reported by Snapper and Grünbaum (1926). Since, in agreement with these workers, we found that 70 per cent of the added β-hydroxybutyric acid was recovered, a correction was applied to the values obtained for this substance in tissue. Blood sugar was determined by the Shaffer-Hartmann method (1920).

TABLE 1

Normal dog

Dog B. Fasted 5 days. Kidneys removed under ether anesthesia. Skin closed 11:00 a.m. Weight 8.5 kilos.

TIME	SUBSTANCE INJECTED	BLOOD SUGAR	BLOOD ACETONE + ACETOACETIC ACID CALCULATED AS ACETONE	BLOOD β-HYDROXY-BUTYRIC ACID CALCULATED AS ACETONE	TOTAL BLOOD KETONE BODIES CALCULATED AS ACETONE
		per cent	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
12:00		0.164	0	0	0
12:00–12:07	8.5 grams acetoacetic acid as Na salt				
12:14		0.142	94.2	16.1	110.0
12:29			72.6	30.8	103.0
12:45		0.111	58.6	59.1	118.0
1:05			38.2	44.5	82.7
1:40			28.4		
2:15			20.8	33.0	53.8
3:15			15.4	15.0	30.4
4:15		0.104	17.0	13.0	30.0
6:20		0.108	16.4	2.5	18.9
6:30	Muscle analysis per 100 grams tissue		13.2	0	13.2

Following its intravenous injection in normal animals, the acetoacetic acid disappeared from the blood, rapidly during the first two hours following its injection and at a much slower rate during the next four hours. β-hydroxybutyric acid made its appearance in the blood immediately after the injection of the acetoacetic acid, then increased in concentration for about an hour or an hour and a half, after which it rapidly decreased. At the end of the 6 hour period of observation, much less β-hydroxybutyric acid than acetoacetic acid was present in the blood. It will also be observed that the greatest amount of β-hydroxybutyric acid was found in

the blood during the period in which the rapid disappearance of acetoacetic acid was taking place.

In a rabbit which received intravenously one gram of acetoacetic acid as the sodium salt per kilo body weight, one-third of one per cent of the administered acetoacetic acid was found excreted in the breath as acetone

TABLE 2

Normal dog

Dog C. Fasted 24 hours. Kidneys removed under ether anesthesia. Skin closed 11:15 a.m. Weight 20 kilos.

TIME	SUBSTANCE INJECTED	BLOOD SUGAR	BLOOD ACETONE + ACETOACETIC ACID CALCULATED AS ACETONE	BLOOD β-HYDROXY-BUTYRIC ACID CALCULATED AS ACETONE	TOTAL BLOOD KETONE BODIES CALCULATED AS ACETONE
		per cent	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
12:00		0.180	0	0	0
12:10–12:15	10 grams acetoacetic acid as Na salt				
12:20–12:27	10 grams acetoacetic acid as Na salt				
12:31		0.188	85.6	27.7	113.0
12:40			71.2	27.0	98.2
12:55		0.177	51.0	29.0	80.0
1:10			43.6	43.5	87.1
1:40		0.156	23.2	46.2	69.4
2:10			17.0	31.0	48.0
3:10		0.117	13.0	6.40	19.4
4:10		0.111	12.8	10.1	22.9
5:10			11.2	6.20	17.4
5:40		0.112	11.2	7.00	18.2
5:52–6:00	15 grams acetoacetic acid intravenously as Na salt		86.8	36.6	123.0
6:05	40 units insulin subcutaneously				
6:15		0.136	63.8	28.0	91.8
6:30		0.133	42.2	30.3	72.5
6:45		0.116	30.0	33.6	63.6
7:15		0.109	20.2	19.6	39.8

(Weber, 1927). This confirms the observations of Friedemann, Somogyi and Webb (1926) who found less than one per cent of the injected acetoacetic acid exhaled as acetone. None of the injected substance was excreted in the urine since the kidneys were removed. Furthermore, no disappearance of acetoacetic acid was detected over a period of 2 hours when this substance in the form of its sodium salt was added to a suspension of dog's

blood in Locke's solution which was kept at 38°C. We may therefore conclude that the disappearance of acetoacetic acid from the blood of the animals reported in this investigation is due to its migration into the tissues of the body.

To ascertain whether acetoacetic acid had been utilized by the organism, it was necessary to determine the amounts of the ketone bodies present in

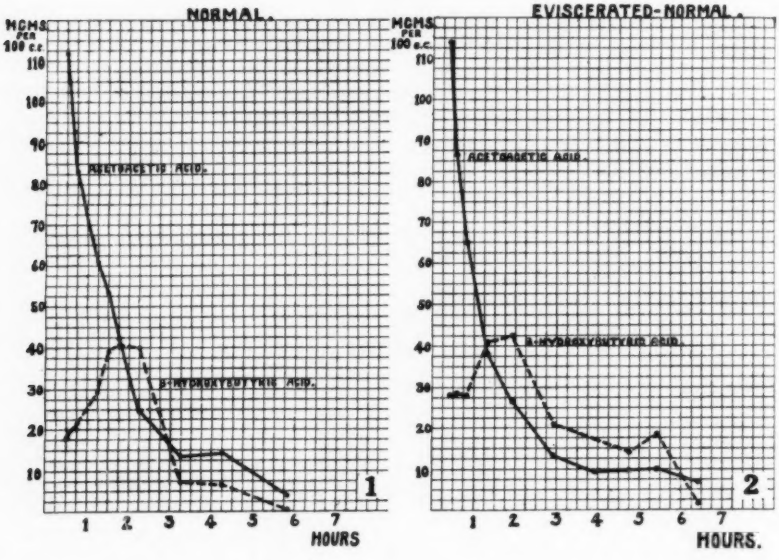

Fig. 1. Dog A. Normal. Fasted 48 hours. Kidneys removed under ether anesthesia. Skin closed 11:38 a.m. Weight, 13 kilo. Twelve grams acetoacetic acid as sodium salt injected at 12:20 p.m.

Fig. 2. Dog D. Eviscerated-normal. Fasted overnight. Kidneys removed and intra-abdominectomy performed under ether anesthesia. Weight, 9 kilos. By 12:20 p.m. animal had received 9 grams acetoacetic acid as sodium salt. Received 45 cc. 10 per cent glucose intravenously at 12:30 p.m. and every 2 hours thereafter. Operation completed 11:00 a.m. and animal in good condition throughout experiment.

the whole body at the end of the experiment. This could be only roughly gauged since analyses were not carried out on all the tissues of the body, and it is improbable that these substances were uniformly distributed throughout the body. For purposes of calculation, it has been assumed, as Bollman, Mann and Magath (1925) have done in their work on uric acid, that the ketone bodies are evenly distributed in 75 per cent of the weight of the eviscerated preparation, and since liver and gastro-intestinal tract

constitute roughly 10 per cent of the body weight, we have further assumed in keeping with the above ratio of distribution that the acetoacetic acid is evenly distributed in 77 per cent of the body weight of the normal animal.

Dog B which was a normal preparation received 8.5 grams acetoacetic acid. At the end of 6.5 hours, the muscles contained 13.2 mgm. of acetoacetic acid calculated as acetone per 100 gram of tissue. Therefore, at the end of this period there was still present in the whole animal $\frac{77}{100} \times \frac{8500}{100} \times \frac{13.2}{1000} = 0.86$ gram acetone or $\frac{0.86 \times 102}{58} = 1.5$ gram acetoacetic acid. Based on such a calculation dog B had utilized about $\frac{8.5 - 1.5}{6.5 \times 8.5} = 0.13$ gram acetoacetic acid per kilo per hour.

Dog C. which was a normal animal, received two injections of acetoacetic acid, one several hours before and the other immediately preceding the administration of 40 units insulin subcutaneously. During the last 45 minutes of the experiment, i.e., between 6:30 and 7:15 p.m. when, as indicated by the falling blood sugar, the insulin was active, the blood acetoacetic acid fell from 42 to 20 mgm. per 100 cc. (calculated as acetone), whereas the β-hydroxybutyric acid of the blood during the same period fell from 30 to 20. By plotting the results of table 2 in a curve, it will be observed that to obtain a similar fall in acetoacetic acid, i.e., 42 to 20 mgm. per 100 cc. blood, required before the administration of insulin, about 45 minutes, and to obtain a fall of β-hydroxybutyric acid from 30 to 20 mgm. per 100 cc. required about 25 minutes. It would appear, therefore, that insulin does not influence the rate of utilization of acetoacetic acid. Similar observations to the effect that insulin does not influence the rate of disappearance of acetoacetic acid from the blood, have been reported by Burn for decapitate cats and by Friedemann, Somogyi and Webb for normal dogs (1926).

The fall of the acetoacetic acid and the rise in the β-hydroxybutyric acid in the blood of the eviscerated preparations are quite similar to those obtained in the normal preparations. In dog E, 5.5 hours after the intravenous injection of 7 grams acetoacetic acid, there still remained in all the tissues $\frac{25.3}{1000} \times \frac{75}{100} \times \frac{7000}{100} \times \frac{102}{58} = 2.3$ gram acetoacetic acid. This animal, therefore, had utilized $\frac{7.0 - 2.3}{5.5 \times 7} = 0.12$ gram acetoacetic acid per kilo per hour.

The results of these experiments show that acetoacetic acid is used by the normal and eviscerated animals at about the same rate, as judged by the disappearance of this substance from the blood. This indicates that the liver and gastro-intestinal tract do not play any important part in the utili-

zation of acetoacetic acid, but that this occurs chiefly in the muscles, which constitute, of course, the predominant active tissue in the eviscerated preparation.

The rate of fall of the acetoacetic acid in the blood is apparently independent of the nutritional condition of the animal, inasmuch as no great variation

TABLE 3

Eviscerated-normal dog

Dog. E. Subtotal ligation of portal vein and vena cava March 11. Intra-abdominectomy April 12. This operation, which was performed under ether anesthesia, was completed by 10:20 a.m. Animal made good recovery and was conscious throughout experiment. Weight 7 kilos.

TIME	SUBSTANCE INJECTED	BLOOD SUGAR	BLOOD ACETOACETIC ACID CALCULATED AS ACETONE	BLOOD β-HYDROXY-BUTYRIC ACID CALCULATED AS ACETONE	TOTAL BLOOD KETONE BODIES CALCULATED AS ACETONE
		per cent	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
11:05		0.065	0	0	0
11:12–11:20	7 grams acetoacetic acid as Na salt				
11:24		0.059	170.0	11.3	181.0
11:39		0.056	116.0	11.0	127.0
12:10		0.034	80.8	20.3	101.0
12:25	4 grams glucose in 20 cc. fluid				
12:35		0.299	75.8	27.2	103.0
1:05		0.153	54.0	32.0	86.0
2:20	4 grams glucose in 20 cc. fluid				
2:30			29.0	35.5	64.5
3:30				34.0	
4:15	4 grams glucose				
4:40			23.4	20.5	43.9
4:40	Muscle analysis mgm. per 100 grams calculated as acetone		20.6	4.7	25.3

in the disappearance of this substance was observed in normal animals according to whether they had fasted 24 hours, 48 hours or 5 days.

PART II: EXPERIMENTS ON DIABETIC AND EVISCERATED-DIABETIC DOGS. *Methods.* Dogs were completely depancreatized. The animals were kept on a diet of raw meat, raw pancreas and sucrose, and were injected twice daily with insulin until the wound had healed. The eviscerations were carried out by removing the gastro-intestinal tract from the abdominal cavity after ligation of the coeliac and mesenteric arteries, and the elimina-

tion of the liver from the circulation by ligation of the portal vein and hepatic artery. This method does not completely exclude the liver from the circulation, since there still remains a small collateral circulation via the diaphragm (Rich, 1923). All animals were fasted and deprived of insulin for 4 days previous to being used in the experiments. By this time it is known that glycosuria and ketosis are prominent symptoms in fasted depancreatized dogs (Chaikoff, 1927). As in the previous experiments, both in the intact and eviscerated preparations, the kidneys were removed after ligation of the renal vessels.

It is strikingly evident from these results (tables 5 and 6 and fig. 3) that the rate of fall of the injected acetoacetic acid was much slower in the blood

TABLE 4

Eviscerated-normal dog

Dog F. Kidneys removed and evisceration completed by 10:45 a.m. Animal made good recovery. Weight 12 kilos.

TIME	SUBSTANCE INJECTED	BLOOD SUGAR	BLOOD ACETOACETIC ACID CALCULATED AS ACETONE	BLOOD β-HYDROXY-BUTYRIC ACID CALCULATED AS ACETONE	TOTAL BLOOD KETONE BODIES CALCULATED AS ACETONE
		per cent	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
11:20		0.135	0	0	0
11:35–11:38	12 grams acetoacetic acid as Na salt				
11:40		0.106	116.0	8.7	125.0
12:10		0.059	51.2	18.3	69.5
12:40			29.8	26.8	56.6
1:00	5 grams glucose in 50 cc. Locke's solution				
1:10		0.236	17.9	30.0	47.9
1:35			9.2	33.2	42.4

of diabetic animals as compared with the normal or eviscerated-normal preparations. The concentration of the β-hydroxybutyric acid also rose slowly in the blood, and its fall after reaching its maximum concentration occurred at a much slower rate than in the normal preparations. In dog K, 6 hours after the administration of 1 gram acetoacetic acid per kilo body weight, 74 mgm. ketone bodies per 100 cc. (calculated as acetone) were still present in the blood, whereas in normal dogs, 5.5 hours after the administration of a similar amount of acetoacetic acid per kilo body weight, the highest concentration found was 18.9 mgm. per 100 cc. blood (calculated as acetone). Again the rate of utilization, as judged by the concentration of these bodies in the muscle, was much less than the normal animals. Assuming that 77 per cent of the weight of the normal animals is soft tissue

in which the acetone bodies are uniformly distributed in a concentration as indicated by muscle, dog L which had received one gram of acetoacetic acid per kilo body weight, had utilized 0.0085 gram per kilo per hour. Friedmann, Somogyi and Webb (1926) found that normal dogs can tolerate almost 4 or 5 mm. sodium acetoacetate per kilo per hour when this substance

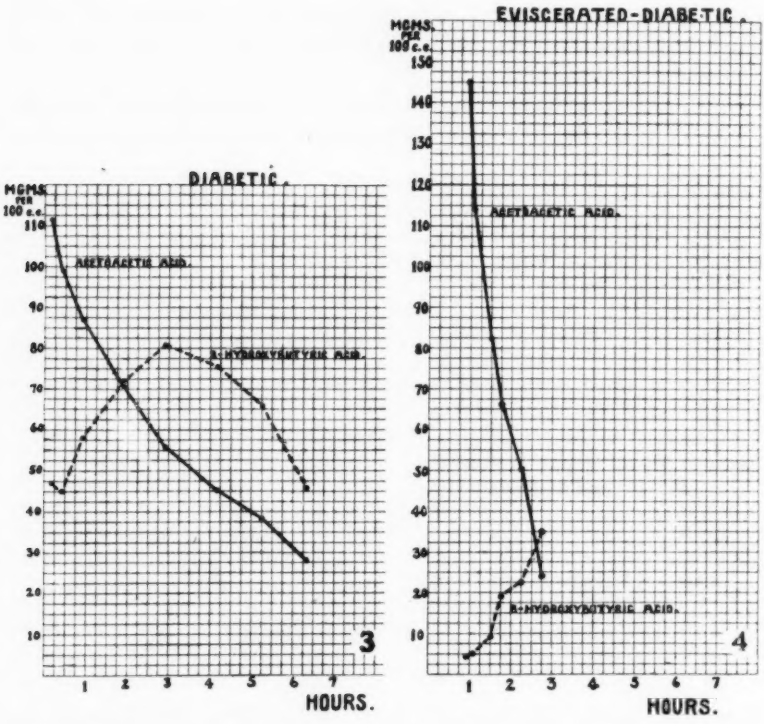

Fig. 3. Dog K. Diabetic. Fasted 4 days. Kidneys removed under ether anesthesia. Weight 6 kilo. Animal received intravenously 6 grams acetoacetic acid as sodium salt.

Fig. 4. Dog P. Eviscerated-diabetic. Fasted 5 days. Eviscerated and afferent liver vessels tied off. Weight 6 kilo. Received intravenously 6 grams acetoacetic acid as sodium salt 12:40–12:50.

is administered intravenously at a constant rate over a long period of time. In two depancreatized dogs which these workers employed, a normal tolerance for this substance was observed. The failure of these workers to detect a difference in tolerance for acetoacetic acid between normal and diabetic dogs, may possibly be ascribed to the method employed, namely, the

slow rate of injection. In the experiments recorded in this paper, in which a decided difference was observed, the animals received intravenously a large amount of acetoacetic acid during a period of several minutes. The disappearance of acetoacetic acid from the blood of the eviscerated-diabetic dogs (tables 7 and 8 and fig. 4) occurred at as rapid a rate as that observed in the normal or normal-eviscerated dogs. Four hours after the intravenous injection of one gram acetoacetic acid per kilo body weight, 31 mgm. of acetone bodies per 100 cc. (calculated as acetone) were still present in the blood of dog Q (table 7). Applying the same method of calculation as before, this dog utilized 0.21 gram acetoacetic acid per kilo per hour.

TABLE 5

Diabetic dog

Dog L. Completely depancreatized. Fasted 4 days. Kidneys removed under ether anesthesia. Weight 9 kilos.

TIME	SUBSTANCE INJECTED	BLOOD ACETOACETIC ACID CALCULATED AS ACETONE	BLOOD β-HYDROXY-BUTYRIC ACID CALCULATED AS ACETONE	TOTAL BLOOD KETONE BODIES CALCULATED AS ACETONE
		mgm. per 100 cc.	*mgm. per 100 cc.*	*mgm. per 100 cc.*
11:15		0	41.0	41.0
3:00		Trace	54.0	54.0
3:08–3:10	9 grams acetoacetic acid as Na salt in 65 cc. fluid			
3:17		93.3	43.5	137.0
3:34		87.0	42.0	129.0
3:55		76.6	46.0	123.0
4:55		76.8	54.5	131.0
5:55		70.8	63.0	134.0
7:45		68.4	44.0	112.0
9:35		34.8	48.0	83.0
9:45	Muscle analysis mgm. per 100 grams calculated as acetone	37.2	32.6	69.8

The results of these experiments show that the muscle of diabetic dogs can utilize acetoacetic acid at a rate which is commensurate with that observed in the normal intact and the eviscerated-normal animals. The fact that the acetone bodies disappear from the blood at a slower rate in the intact diabetic dogs, than in the normal animal can, therefore, not be attributed to decreased utilization by the muscles. The work of Fischler and Kossov (1913) who found that ketosis was much reduced in phlorhizinized animals following establishment of an Eck fistula and ligation of the portal vein, points to the liver as the seat of formation of the ketone bodies. Almagia and Embden (1905) first showed that the liver when perfused with

its own blood produces acetone bodies whereas these substances do not appear in the perfusates of the other tissues of the body (Embden and Kalberlah, 1906). It would seem, therefore. that the ketone bodies produced by the liver of the fasted depancreatised dog is the explanation for the difference observed in the rates of disappearance of acetoacetic acid from the blood of intact and eviscerated-diabetic preparations. In other words, the rate of utilization of acetoacetic acid in the diabetic dog is probably the same as in normal animals, but as judged by the blood, there is an "apparent decrease" in the rate of utilization of the injected substance in the former, due to the fact that the ketone bodies produced in the liver are being added to the blood at the same time that the injected acetoacetic acid is being used by the tissues. Evidence for this view is provided by

TABLE 6

Diabetic dog

Dog M. Completely depancreatized. Fasted 4 days. Kidneys removed under ether anesthesia. Weight 12 kilos.

TIME	SUBSTANCE INJECTED	BLOOD ACETOACETIC ACID CALCULATED AS ACETONE	BLOOD β-HYDROXY BUTYRIC ACID CALCULATED AS ACETONE	TOTAL BLOOD KETONE BODIES CALCULATED AS ACETONE
		mgm. per 100 cc.	*mgm. per 100 cc.*	*mgm. per 100 cc.*
11:55			24.0	24.0
12:00–12:10	12 grams acetoacetic acid as Na salt in 160 cc. fluid			
12:16		116.0	26.2	142.0
12:31		97.8	29.2	127.0
1:00		85.2	41.0	126.0
1:45		75.8	48.5	124.0
2:45		65.4	59.0	124.0
3:45		68.0	72.5	140.0
4:45		59.7	62.0	122.0

dog L in which the concentration of the ketone bodies in the blood previous to the injection of acetoacetic acid rose from 41 to 54 mgm. per 100 cc. in 155 minutes. In another nephrectomized, depancreatized dog which had fasted four days, the ketone bodies in the blood increased by 13 mgm. per 100 cc. (calculated as acetone) at the end of five hours. In the light of the facility with which the eviscerated-diabetic animal is capable of utilizing acetoacetic acid, the increased amounts of ketone bodies found in these cases in the blood is but a small index of the amount of this substance actually produced by the liver during this period.

Since the essential difference between the eviscerated-normal and eviscerated-diabetic preparations is the presence of insulin in the tissues of the

normal animal, it is again evident, as already noted above, that insulin can play but an unimportant part in the rate of utilization of acetoacetic acid.

DISCUSSION. From the foregoing experiments it is evident that acetoacetic acid is readily utilized by muscle, and it must, therefore, be regarded as an available source of energy in the animal organism, particularly during fasting or as a result of removal of the pancreas or following the ingestion of a carbohydrate-free or high fat diet, under which conditions the ketone bodies make their appearance in increased amounts in blood and urine. These observations also suggest that the ketone bodies are normal inter-

TABLE 7

Eviscerated-diabetic dog

Dog Q. Completely depancreatized. Fasted and deprived of insulin for 4 days. Gastro-intestinal tract removed and liver eliminated from the circulation by ligation of portal vein and hepatic artery. Kidneys removed. Operation completed by 10:25 a.m. Weight 14 kilos.

TIME	SUBSTANCE INJECTED	BLOOD SUGAR	BLOOD ACETOACETIC ACID CALCULATED AS ACETONE	BLOOD β-HYDROXY-BUTYRIC ACID CALCULATED AS ACETONE	TOTAL BLOOD KETONE BODIES CALCULATED AS ACETONE
		per cent	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
11:30			0	17.3	17.3
11:40–11:44	14 grams acetoacetic acid as Na salt in 150 cc. fluid				
11:49			88.6	20.3	109.0
12:05			57.6	28.0	85.6
12:37		0.283	44.4	20.5	64.9
1:05			33.8	16.4	50.2
2:05		0.198	22.1	31.3	53.4
3:05			18.2	24.2	42.4
3:50			13.5	17.4	30.9
4:05	Muscle analysis mgm. per 100 grams tissue		12.0	0	12.0

mediary metabolites, but that they escape detection in anything but small amounts in the blood of normal animals, because of their rapid utilization by muscle. Marriot (1913) has reported that the ketone bodies are present in the blood of human subjects and of normal dogs, in a concentration as high as 40 mgm. per liter.

As to the fate of the acetoacetic acid which disappears in the animal organism, little that is definite is known. In the eviscerated spinal cat, it is not condensed to glycogen. It was found that acetoacetic acid led to an increased O_2 consumption when administered to fed and fasted animals.

Knoop and Jost (1924) found lactic acid excreted in the urine following administration of β hydroxybutyric acid, but in view of the fact that it would entail an oxidation in the β position, these workers do not conclude that the lactic acid is derived from the β hydroxybutyric acid. Shaffer's *in vitro* experiments suggest a reaction between the ketone bodies and glucose or one of its products, but Ernst and Forster (1924) have shown that mannitol, glycerol, mono- and tributyrin are just as capable of accelerating the *in vitro* disappearance of acetoacetic acid in the presence of hydrogen peroxide.

As much as 2 grams of the ketone bodies may be excreted in the urine by completely depancreatized dogs on the fourth day of fasting. Since our experiments show that on the fourth day of fasting the muscles of the depancreatized dogs can utilize acetoacetic acid as rapidly as normal muscle,

TABLE 8

Eviscerated-diabetic dog

Dog R. Completely depancreatized. Fasted and deprived of insulin for 4 days. Procedure as in table 7. Operation completed by 2:45. Weight 14 kilos.

TIME	SUBSTANCE INJECTED	BLOOD SUGAR	BLOOD ACETOACETIC ACID CALCULATED AS ACETONE	BLOOD β-HYDROXY-BUTYRIC ACID CALCULATED AS ACETONE	TOTAL BLOOD KETONE BODIES CALCULATED AS ACETONE
		per cent	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*mgm. per 100 cc.*
3:05		0.270	0	7.3	7.3
3:12–3:16	14 grams acetoacetic acid as Na salt in 125 cc. fluid				
3:21			156.0	28.5	184.0
3:40			110.0	34.4	144.0
4:25		0.277	64.0	37.0	101.0
5:25			0.0	60.5	60.5

ketosis can not be attributed to an impediment in the normal path of the catabolism of the ketone bodies. We can, therefore, draw the conclusion that the appearance of the ketone bodies in excessive amounts in tissue fluids is due to increased rate of production by the liver.

As to the mechanism whereby insulin affects its antiketogenic action, it is clear from our experiments and from those of Burn (1925) and of Friedemann, Somogyi and Webb (1926) that it does not do so by influencing the rate of utilization of acetoacetic acid. Since the glucose excreted by the fasted depancreatized dog is, in part at least, derived from fatty acid, the appearance of glucose and of ketone bodies in abnormal amounts in the urine of fasted depancreatized dogs is due to the same process, namely, one involving the excessive conversion of fat to carbohydrate and acetone bodies,

and it is by inhibiting this process of gluconeogenesis from fat that insulin effects its antiketogenic action.

The fact that glucose and the ketone bodies may have a common source and make their appearance in blood and urine of the diabetic animal under the same metabolic disturbance, indicates that a close relationship exists in the intermediary metabolism of these substances. A similar view was pointed out several years ago when, following the administration of insulin to fasted depancreatized dogs, a striking parallelism was observed in the fall of the glucose and of hydroxybutyric acid in the blood (Chaikoff, Macleod et al., 1925).

SUMMARY

Following its intravenous administration, sodium acetoacetate disappears rapidly from the blood of normal animals and eviscerated-normal animals. Since the acetone bodies present in the tissues of the latter preparation several hours after its injection will not account for the acetoacetic acid injected, the conclusion is drawn that muscle is capable of utilizing acetoacetic acid.

The fall of acetoacetic acid in the blood and tissues of fasted depancreatized dogs occurs at a slower rate as compared with normal animals, whereas the disappearance of this substance from the blood and tissues of diabetic dogs upon which evisceration had been performed, occurs as rapidly as in normal animals.

The conclusion is drawn that the appearance of the ketone bodies in excess amounts in the tissue fluids of the diabetic animal is not due to a deficient utilization of these substances by the organism.

The further application of these results to the mechanism of ketogenesis is pointed out.

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UNIFORMITY OF NARCOSIS IN PERIPHERAL NERVE

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Most of the evidence supporting the theory of "conduction with a decrement" in narcotized nerve has been satisfactorily explained without the hypothesis of decrement. Since this evidence has been recently reviewed by one of us (Davis, 1926) it is unnecessary to discuss it further here. However, the increase in the least interval for muscular summation with increasing length of narcotized nerve is still a disputed point.

If two successive stimuli are applied to a motor nerve a summated muscular response results, provided the second stimulus falls outside the absolute refractory period of the first impulse. The smallest time interval separating two stimuli which produce summation is known as the "least interval for muscular summation." Adrian and Lucas (1912) and Lucas (1913) observed an increase in the least interval when stimuli are applied to a narcotized region of nerve and also a correlation of this increase of interval with the length of narcotized nerve which the impulse must traverse. Such a lengthening of the least interval can be explained either by *a*, decremental conduction, assuming that the second impulse, arising as early as ever in the refractory period, would be too small to pass the region of decrement resulting from narcosis, or *b*, by a slowed recovery process which would lengthen the absolute as well as the relative refractory period. Adrian and Lucas believed that the rate of recovery was unchanged by alcohol narcosis and argued for decremental conduction.

Kato repeated Lucas' work with more perfect controls and found no correlation of least interval with length of nerve. He observed a marked but uniform increase in least interval at all points stimulated. In his second monograph he presents direct evidence for slowed recovery in narcosis by showing that the least interval for muscular summation may actually become greater than the time necessary for complete recovery in normal nerve.

Cooper (1926) also repeated Lucas's experiments on least interval. She used three slot electrodes, B, C and D. In her early experiments she used alcohol as a narcotic, but later substituted chloral hydrate, as its action seemed less variable than that of alcohol. In all 16 experiments reported her results agree with those of Lucas in that they show consistently a

greater "least interval" and an earlier failure of conduction at the upper electrode, D, than at C, and in turn an earlier failure at C than at B. In her experiments with chloral hydrate as a narcotic the least interval at D began to increase, on the average, 10 minutes prior to that at C and the interval at C 12 minutes prior to that at B.

In Cooper's experiments a 35.5 mm. length of nerve was narcotized in a chamber of the slot electrode type used by Lucas (1913). Branching areas were included in this narcotized region. It was therefore possible that histological differences might lead to greater penetrability of one region of nerve, for example the upper thigh region where branches are given off, than another. Cooper states that "during the course of a considerable number of experiments nerves of all sizes have been used and it has never seemingly made any difference if the electrodes have been directly on the part where branches are given off" (p. 317). These incidental observations of Cooper's on penetration of narcotic into nerve as affected by size and branching of the nerve trunk seemed to us inadequate. It was further to investigate such possible causes of variation in the penetration of narcotics into nerve that the present research was undertaken.

Penetration of narcotic as affected by diameter of the nerve trunk. The effect of diameter on the penetration of narcotic into a nerve trunk was investigated in ten large, native bull-frogs. The two gastrocnemius nerve-muscle preparations from a single frog were dissected out and cleaned of all superficial fascia. Regions of different size, i.e., the 7th roots, the 8th roots, the lower sciatic distal to the thigh branches, and finally the popliteal branch to the gastrocnemius muscle, were mounted symmetrically and narcotized simultaneously in the same 12 mm. narcotizing chamber. Three per cent chloral hydrate was used throughout as a narcotic.

The time required to establish a complete block was plotted against the diameter of the respective nerve. No clear correlation for any single region appeared, although the small popliteal region failed somewhat earlier than the larger spinal roots or the main trunk of the sciatic.

Narcosis of "central" and "distal" thigh regions. Sixty-eight experiments were next done to determine if there was any inherent difference in the penetrability of the two regions of the sciatic nerve designated by Cooper as "upper" and "lower" and herein referred to as "central" and "distal." The central region of one preparation, i.e., that portion between the spinal root junction and the thigh branches, and the distal region of the other preparation, i.e., that unbranched portion of nerve just distal to the thigh branches, were mounted as above, in an 8 x 8 mm. chamber. This size was used instead of the 12 x 12 mm. chamber owing to difficulty in obtaining nerves with a 12 mm. central region free from branches.

Eighteen experiments were performed in the autumn on native frogs. In 14 of these failure occurred first in the nerve narcotized centrally. After

a lapse of a month 25 experiments were done on winter frogs from various sources with a result of 14 to 11 in favor of earlier central failure. It was thought that the type of frog or time of year might be the cause for the difference in ratio of central-distal failure. In the spring a third series was done with a resulting score of 6 to 19 in favor of distal failure. This made the total score for the entire series 34 to 34, a 1 to 1 ratio. We therefore

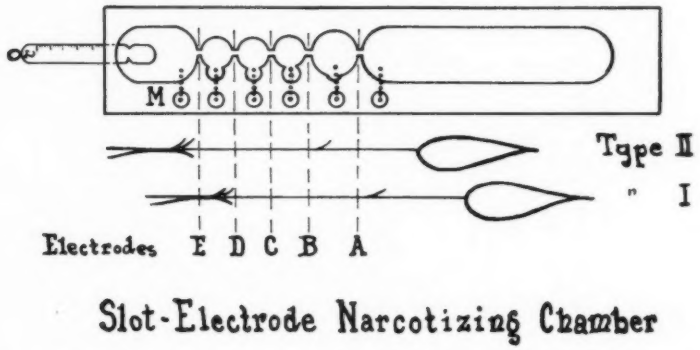

Fig. 1. Plan of narcotizing chamber, showing relationship of branches to slots in the two types of mounting. Dotted lines indicate silver-silver chloride electrodes from mercury cups, M, to fluid chamber. Distance between slots *A–B*, 12.5 mm.; *B–C*, *C–D* and *D–E*, 9.5 mm. The chamber is provided with a writing lever (not shown) as in Lucas' original design.

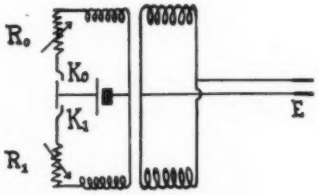

Fig. 2. Wiring plan of Lucas pendulum for delivery of double stimuli at varying intervals. K_0, stationary key; K_1, adjustable key; E, stimulating electrodes; R_0 and R_1, resistances for varying the strength of stimuli.

consider that there is no evidence of a greater inherent penetrability of the sheath in the upper thigh region, as compared with the lower.

At about this time a communication was received from Kato (1927). He and his co-workers reported experiments to show that, unless the dissection of a nerve-muscle preparation be done with the greatest care to avoid unconscious mechanical injury, narcosis will proceed more rapidly at points

TABLE 1

Tabulation of experiments with type I mounting

A, experiments giving progressive cessation of conduction from successive electrodes. B, experiments giving simultaneous cessation of conduction from D and E, but progressive cessation from lower electrodes. Three per cent chloral hydrate Ringer's solution was used.

	EXPERIMENT NUMBER	TEMPERATURE	DIFFERENCE IN TIME OF FAILURE				POSITION OF ELECTRODES IN RELATION TO BRANCHING
			D after E		C after D		
A							
			minutes	*per cent*	*minutes*	*per cent*	
December 5, 1927	1	18–19	12′ 50″	13.5	4′ 20″	4.4	E central region D distal to thigh branch
March 5, 1928	2	19±	2′ 30″	4.2	1′ 50″	2.5	E central D thigh branch
March 9, 1928	3	18.5	5′ 50″	15.0	2′ 20″	5.3	E central D thigh branch
March 14, 1928	4	18–18.5	2′	2.2	1′ 30″	1.7	E central D thigh branch
Average			5′ 50″		2′ 30″		

B

November 28, 1927	5	21	Simultaneous	8′ 10″	14	E central region D distal
December 6_{II}, 1927	6	19	Simultaneous	5′ 20″	11	E spinal roots D central region
March 12_{II}, 1928	7	18	Simultaneous	5′	6	E central D thigh branches
March 12_{I}, 1928	8	18.5	Simultaneous	10′	14	E central D thigh branches
March 14_{II}, 1928	9	19.0	Simultaneous	3′	3.7	E spinal root junction D thigh branches
Average				6′ 18″	9.9	

of branching. They found by hanging weights of varying magnitude on the nerve that a tension of even one gram may hasten the onset of narcosis. Such mechanical injuries, they claim, are among the sources of error which have led to the theory of conduction with a decrement.

We then carried out controls for equal dissection, in which both preparations were stripped of equal amounts of the grosser outer fascia as usual and were mounted to narcotize similar regions. Conduction ceased simultaneously in the two preparations or within 6 per cent of each other. But

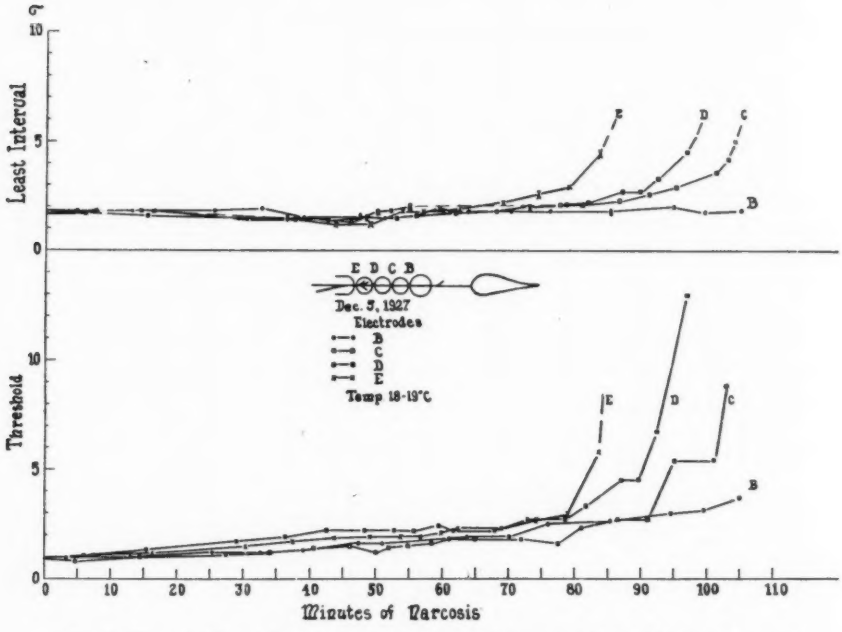

Fig. 3. Threshold and least interval for muscular summation with type I mount showing progressive increase in least interval at successive electrodes.

when one nerve was stripped of all fascia instead of only the outer layer, such a nerve failed about 40 per cent earlier than a standard preparation. These observations are in keeping with Kato's, although it is difficult to say how much of the increased penetrability is due to trauma and how much to the decreased amount of fascia on the "supercleaned" nerve.

Least interval for muscular summation. Our experiments so far showed no justifiable grounds for criticizing Cooper on the basis of differences in penetrability due either to the size of the region narcotized or to histological differences in the central and distal regions of unbranched nerve. But a

probable explanation of Cooper's results seemed to be a greater penetrability at regions of branching. We, therefore, repeated Cooper's experiments on least interval for muscular summation, noting the variations in the results which were correlated with the position of the branches in relation to the stimulating electrodes.

METHOD. A bull-frog's gastrocnemius nerve-muscle preparation was dissected, cleaned as usual and mounted in a slot-electrode narcotizing chamber of the Lucas type (1913) with the same dimensions as Cooper's

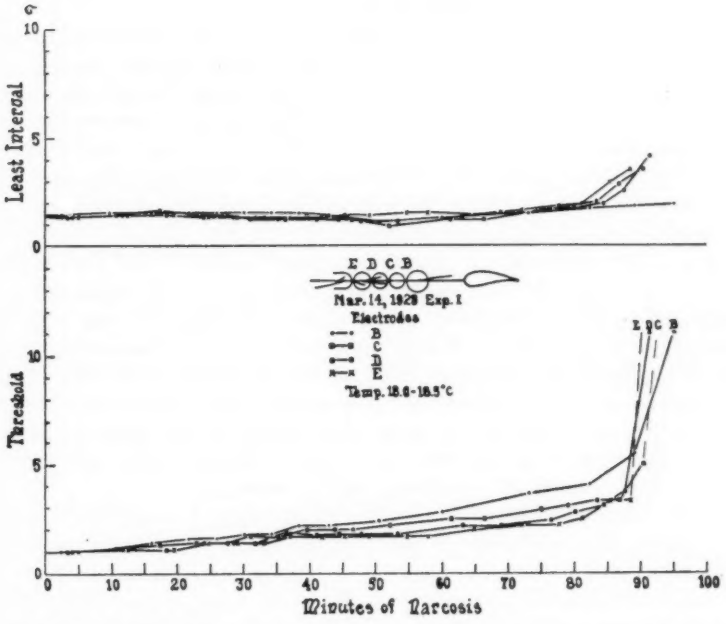

Fig. 4. Threshold and least interval for muscular summation with type I mount, showing a nearly simultaneous increase in least interval at all electrodes. Special care was taken in this dissection to avoid trauma; cut ends were also ligated.

(fig. 1). Slot A was sealed with vaseline and the entire chamber was filled with Ringer's solution. A Lucas pendulum was arranged to deliver two stimuli at varying intervals. Figure 2 shows the wiring plan with resistance boxes for varying the strength of the stimuli. The thresholds and least intervals at slots B, C, D and E were recorded two or three times at ten minute intervals, to be assured of an approximately steady condition of the nerve prior to starting narcosis. The Ringer's solution above slot A was then replaced by Ringer's solution containing 3 per cent chloral hydrate.

In general two types of mounting were employed. In type I (fig. 1) electrode E was either at the distal region between the thigh branches and the spinal roots, or at the spinal root junction; the peroneal branch of the sciatic nerve was always just outside slot A (i.e., outside the region of narcosis); electrode D was at the region of thigh branching or just central to it; C was, with but one exception, at the distal, unbranched region and B always at this distal region of nerve. In type II (fig. 1) electrode E was just distal to the region of thigh branching, D and C were always in the unbranched distal area and the peroneal branch of the sciatic fell midway between either slots A and B or B and C.

Results. The results of the experiments with type I mounting may be divided into two groups. The first of these groups comprises four experiments (table 1A) and confirms Cooper in that failure always occurred in the order of distance of the electrodes from the muscle. The average time of failure of conduction from E was 5′5″ before that from D and D failed an average of 2′30″ prior to C. Experiment 1 on December 5 (fig. 3) shows this behavior most markedly; the least interval at E diverged from that at D 65 minutes after narcosis was begun, the interval at D diverged from that at C 15 minutes later. Conduction from E failed 12′50″ earlier than from D and from D 4′30″ earlier than from C. In all experiments B continued to evoke responses, as in Cooper's experiments, long after no response could be obtained from other electrodes. This was undoubtedly due to a spreading of the stimulating current to normal nerve beyond the narcotized region. The results of this group of experiments, then, not only confirm Cooper's results with three electrodes, but the behavior of our fourth electrode, E, is, according to her interpretation, further evidence in support of the theory of conduction with a decrement.

In the other five experiments (table 1B) with type I mounting, conduction ceased from electrodes D and E at the same time. This may have been due to a simultaneous cessation of function at both electrodes or to an earlier failure at D than at E. In the latter case the actual least interval for E itself could not be followed, since the impulses set up here would be blocked at D. Electrode C, however, behaved in all five experiments as it did in Cooper's experiments; it failed an average of 6′18″ later than D, and conduction was still good from B, due to current spread, when no response was elicited from C.

It should be noted, however, that experiments 4 (fig. 4) and 9, both of March 14, 1928, show much less difference in the time of failure at the various electrodes than do the other experiments of this type. These two experiments were performed after the type II group described below and in them special care was taken in dissection to give the preparation a minimum amount of handling. Only the grosser, outer fascia was removed, the sheath in the branching regions was split no more than was necessary, and the cut ends of the branches were ligated.

Experiments with type II mounting (table 2) are in marked contrast to those of type I. Conduction failed simultaneously or very nearly so from all three upper electrodes. But the curves for least interval at electrodes C, D and E in experiments 10 to 13 are all characterized by an abrupt break, the interval rising suddenly to infinity as conduction from these electrodes ceased. This can only mean that a block occurs somewhere between electrodes C and B. In this type of mounting it may be recalled that the peroneal nerve is the only cut branch in the range of electrodes A to E and that it invariably lay between A and C. In view of Kato's report it seemed

TABLE 2

Tabulation of experiments with type II mounting

Three per cent chloral hydrate in Ringer's solution was used except in the experiment of February 20, 1928, in which a 4 per cent solution was used.

DATE	NUMBER	TEMPERATURE	POSITION OF ELECTRODES IN RELATION TO BRANCHING
		°C.	
November 25, 1927	10	20±	No divergence of C and D, which failed simultaneously, from B
November 26, 1927	11		No divergence of C, D and E which failed simultaneously, from B
November 28, 1927	12	21.5	C, D and E failed together and diverged from B 6′ prior to their failure
December 6, 1927	13	18–19	C, D and E failed together and diverged from B 8′ prior to their failure
January 30, 1928	14	15.2	C, D and E failed together
February 1, 1928	15	18±	C, D and E failed together
February 6, 1928	16	19.5	C, D and E failed together
February 20, 1928	17	20.5	C, D and E failed together
March 2, 1928	18	19.5	D and E failed together, C 50″ later; difference is 1.4 per cent
March 5, 1928	19	19.5	D and E failed together, C 2′ 50″ later

likely that this sudden failure was due to damage at this branch. Experiments 14 to 19 were done using exceptional care in dissection to avoid trauma. The nerve was carefully cleaned as usual, but a 1 cm. instead of 0.5 cm. length of the branching peroneal was left, and the sheath at the point of branching was not split as far as usual. The results of experiment 14 are shown in figure 5. The least interval increases gradually but equally at all three upper electrodes; the curve for B shows only a slight rise, which points to a spread of stimulating current. The results of experiments 15 to 17 are similar to those of experiment 14. In the last two experiments, those of March 2 and 5, 1928, conduction ceased at C not more

than 50 seconds in the one case and 2′50″ in the other, later than at D and E, which failed simultaneously. The difference in the first case is hardly more than the experimental error. In the second case it may have been due to current spread, which occurred occasionally from this electrode.

Two experiments on the least interval for muscular summation were performed on the unbranched peroneal nerve of the cat. The muscle was left intact so that its circulation was undisturbed; otherwise the technique was the same as in the frog experiments. The least interval increased

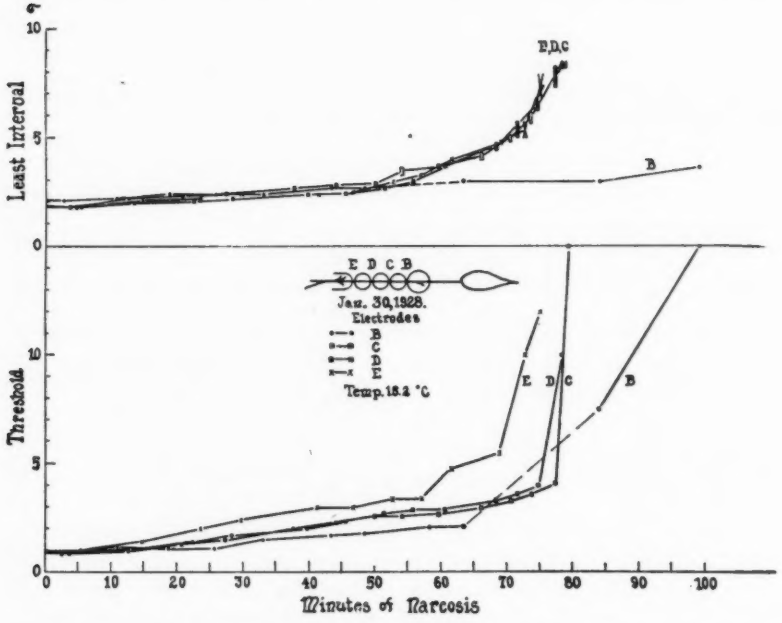

Fig. 5. Threshold and least interval for muscular summation with type II mount, showing a simultaneous increase in least interval at all electrodes.

nearly equally at the three upper electrodes and failure of conduction was approximately simultaneous.

Discussion. Four experiments, numbers 14 to 17, with type II mounting, and in which special care was taken to avoid injury during dissection, especially at branching regions, definitely confirm Kato's results on least interval, since they show an equal increase in interval at all electrodes, and conduction from all electrodes failed simultaneously. Experiments 18 and 19 are also in keeping with Kato's results. Of the nine experiments with type I mounting, four completely confirm Cooper, i.e., they show pro-

gressive but unequal increases in the least interval at successive electrodes, the interval increasing more rapidly the farther the electrode is from the muscle. The results of the other five type I experiments were partly like Kato's in that a simultaneous increase in least interval and failure of conduction occurred at D and E, but partly like Cooper's, since the least interval for D diverged from the base-line earlier than at C, and conduction failed earlier at the upper electrode, D, than at C. Thus either Cooper's or Kato's results may be reproduced by mounting the nerve in one of two different ways, either including the branching area of the thigh region, or avoiding this branched region.

However, it is shown in experiments 4 and 9 that the Cooper type of mounting may give results approaching the Kato type, provided special care is taken to avoid injury during dissection. Our evidence, therefore, indicates that the Cooper type of result, in which the increase in least interval is greater the farther up the nerve, is due to injury at branches lying between the electrodes, since by eliminating such regions or by using sufficient care in dissecting a branched area, Kato's type of result is obtained. This conclusion is confirmed by the experiments on cat nerves, the results being of the Kato type as would be predicted from the uniformity of the nerve exposed to narcotic.

The experiments of Lucas and of Cooper which showed a correlation of increase in least interval with the length of nerve narcotized are therefore untrustworthy, since either the narcotic probably penetrated more rapidly at points of branching or the nerve here was more susceptible to narcosis because of the mechanical trauma of dissection. The anatomy of the frog's sciatic nerve, bringing nerve branches regularly in the neighborhood of the more distant electrodes of Lucas' apparatus, apparently gave rise to an illusion of decremental conduction. We confirm Kato in his conclusion that in uniformly narcotized nerve the least interval for muscular summation increases equally at all points and affords no evidence in support of the theory of "conduction with a decrement."

SUMMARY

1. The time required for narcosis of a 12 mm. length of various parts of sciatic nerve of the bull-frog is not clearly correlated with the diameter of the nerve, except that the small branch to the gastrocnemius fails earlier than the main trunk.

2. In sixty-eight experiments the two sciatic nerves from a single frog were narcotized simultaneously; one centrally to the thigh branches, the other distally. Thirty-four of these showed an earlier central than distal failure; the remaining thirty-four showed an earlier distal than central failure. There appears to be no outstanding difference in the penetrability of these two regions to chloral hydrate.

3. Nerves deprived of only the loose outer sheath of fascia and narcotized in corresponding regions showed either a simultaneous failure or a difference in narcosis rate of not more than 6 per cent. Preparations stripped as cleanly as possible showed a greatly increased narcosis rate. It is difficult to estimate how much of this increased susceptibility is due to trauma and how much to more rapid penetration of narcotic as the result of decreased fascia.

4. Cooper's experiments relating the least interval for muscular summation to the length of nerve narcotized were repeated on large bull-frogs, and her results were duplicated in most experiments in which the branching thigh region was included in the narcotized region. But 6 experiments in which this branching region was avoided, 2 experiments in which the branching region was included but dissected with unusual care, and 2 experiments on the (unbranched) cat's peroneal nerve showed no correlation of least interval with length of nerve.

5. Our experiments confirm Kato in showing a uniformity of the least interval for muscular summation when uninjured nerve is narcotized uniformly. Lucas' and Cooper's apparent correlation of the increase in least interval for muscular summation with the length of nerve exposed to the narcotic presumably depends on the presence of branches and mechanical trauma in dissection.

6. These observations constitute further evidence supporting the theory of decrementless conduction in narcotized nerve.

We wish to thank Miss S. Colt for assistance in the preparation of the manuscript.

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STUDIES IN THE PHYSIOLOGY OF VITAMINS

VI. The Effect of Insulin on Gastric Motility in Vitamin B Deficiency[1]

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A number of investigators (Funk and von Schönborn, 1914; Collazo, 1922–23; Shinoda, 1924; Drummond and Marrian, 1926) have described the occurrence of hyperglycemia in B-avitaminosis. Cowgill et al. (1926) reported that there is a decrease in the vigor of hunger contractions and usually gastric atony in the advanced stages of vitamin B deficiency in dogs. Bulatao and Carlson (1924) have shown that insulin injected into normal dogs augments gastric motility. The question naturally arose whether or not the hyperglycemia reported as occurring in vitamin B deficiency was in any way related to the disturbances in gastric motility, and whether insulin injections could reëstablish hunger contractions in those vitamin B deficient dogs which developed gastric atony, either partial or complete.

Experimental. Apparently normal dogs in good nutritive state, weighing between six and fourteen kilos were kept under observation for about two weeks. During this time attention was paid to their general activity, desire to partake of the artificial ration, and the presence or absence of worms in the stools after administering vermifuge and castor oil. Only vigorous, active animals which seemed particularly suited for these experiments were chosen.

The dogs were fed a mixture of isolated food substances, diet III (Cowgill, 1923), complete in every known respect except for vitamin B.[3] They were given an amount of this food mixture daily which was adequate to

[1] The data in this paper are taken from a dissertation submitted by Charles J. Stucky in partial fulfilment of the requirement for the degree of Doctor of Philosophy, Yale University, 1927.

[2] Porter Fellow of the American Physiological Society, 1926–27.

The authors are grateful to Mr. Robert A. Frisch and Miss Tony Liebman of the Yale University School of Medicine for valuable assistance rendered during the progress of some of the experiments.

[3] In this paper, the term vitamin B refers to the combination of two or more factors which have been shown to make up this substance (Smith, 1928).

maintain a constant body weight. Cod-liver oil was administered at frequent intervals to insure an abundant supply of fat-soluble vitamins. The vitamin B complex was supplied in the form of vitavose[4] (Hoffman, 1925), a wheat germ preparation, during the observation and recovery periods. When rapid recovery from "polyneuritic" symptoms was desired, other sources of vitamin B such as yeast vitamin powder (Harris)[5] and "Vegex"[6] were used as therapeutic agents.

Simple gastric fistulae of the valve type were prepared in seven dogs. The animals were trained to lie quietly and hunger contractions were recorded by the balloon method of Carlson (1916). Tracings were made on an "endless" roll of white paper, as described by Cowgill and his associates (1926). The technique was slightly modified to permit the *recording of the gastric contractions of four dogs simultaneously.* Records were always taken from fifteen to twenty hours after food was offered. Tracings of gastric motility were taken frequently on each dog during the progress of vitamin B deficiency and during the recovery period after vitamin B therapy had been instituted.

The effect of insulin[7] injections on the motility of the empty stomach was studied during the advanced stages of this deficiency, when a decrease in motility occurred, and during the recovery period before the normal sequence had been reëstablished.

Blood sugar was estimated by the method of Hagedorn and Jensen (1923). Hemoglobin was estimated colorimetrically. The earlier determinations were made by the Newcomer method (1919) but later a modification of the Cohen and Smith technique (1919) was employed. Percentages are expressed on the Haldane scale. All determinations of the blood constituents were made in duplicate.

RESULTS. Five of the animals employed manifested a distinct decrease in gastric motility during vitamin B deficiency, while two showed an augmentation of the hunger contractions. The effect of insulin on the stomach contractions of dogs in advanced stages of vitamin B deficiency is summarized in table 1. In the case of dog 1 (experiment 2), the injection of insulin produced a marked increase in gastric tonus and fairly vigorous contractions. This is clearly depicted in figure 1.[8] Vigorous contractions

[4] Obtained through the courtesy of the Ward Baking Company, New York, N. Y. and E. R. Squibb and Sons, New York, N. Y.

[5] From the Harris Laboratories, Inc., Tuckahoe, New York.

[6] This is a highly concentrated vegetable extract made from grain grown yeast and prepared with all the salts of sea water. It contains no animal matter. It may be obtained from Vitamin Food Co., Inc., Westfield, Massachusetts.

[7] Iletin (insulin, Lilly) obtained from the Eli Lilly and Company, Indianapolis, Indiana, was used in these experiments.

[8] This chart was prepared by tracing the original record of the gastric contractions over carbon paper on heavy white drawing paper and then re-tracing with India ink. The chart was then reduced by photography, the original chart being 25 by 38 inches.

TABLE 1

TABLE 1

Résumé of insulin experiments

EXPERIMENT NUMBER	DOG	DAYS ON VITAMIN B DEFICIENT DIET	CONDITION OF ANIMAL	INSULIN INJECTED	BLOOD SUGAR		DECREMENT IN BLOOD SUGAR	RESULTS AND REMARKS
					Initial	Lowest		
				units per kilo	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*per cent*	
1	3	48	"Polyneuritic symptoms"	10	120			Convulsions within 10 minutes and death later
2	1	52	Somewhat uncertain gait; ate all food until 50th day	2	131	57	56.5	Vigorous contractions; see chart
3	2	55	No symptoms; ate all food until 53rd day	0.5	124	77	37.9	No apparent effect
4	2	58	No symptoms	0.5	95	53	44.2	No apparent effect
5	4	58	Animal active; appetite erratic	0.5	93	57	38.7	No apparent effect (protocol 23)
6	2	60	Nervous symptoms; gait uncertain	1	112	57	49.1	Improvement in gastric tonus and small isolated contractions
7	4	60	Active; appetite erratic	1	89	53	40.4	No apparent effect
8	2	62	Muscles spastic; can not walk	1.5	139	109	21.6	No apparent effect
9	4	62	Erratic appetite	1.5	109	58	46.8	No effect; animal died on following day
10	5	65	Paresis	3	137			No effect. Convulsions and death 2.75 hours after insulin injection
11	8	75	Tender skin; no obvious symptoms	2	95	60	36.8	No change
12	8	76	Tender skin; no obvious symptoms	4	108	51	52.8	No definite response
13	6	76	Erratic appetite; somewhat less active; definite anemia*	2	121	63	47.9	Very vigorous contractions and marked tonus variations (protocol 31)
14	6	78	Nervous symptoms; given vitamin B at end of insulin experiment	2	99	70	29.3	No apparent effect
15	6	4†	Appetite good; gait improved	2	115	53	53.9	Vigorous contractions and pronounced tonus changes (protocol 33)
16	6	6†	No definite symptoms	2	122	46	62.3	No effect

* Anemia persisted until death on 89th day.

† Number of days after vitamin B therapy was begun.

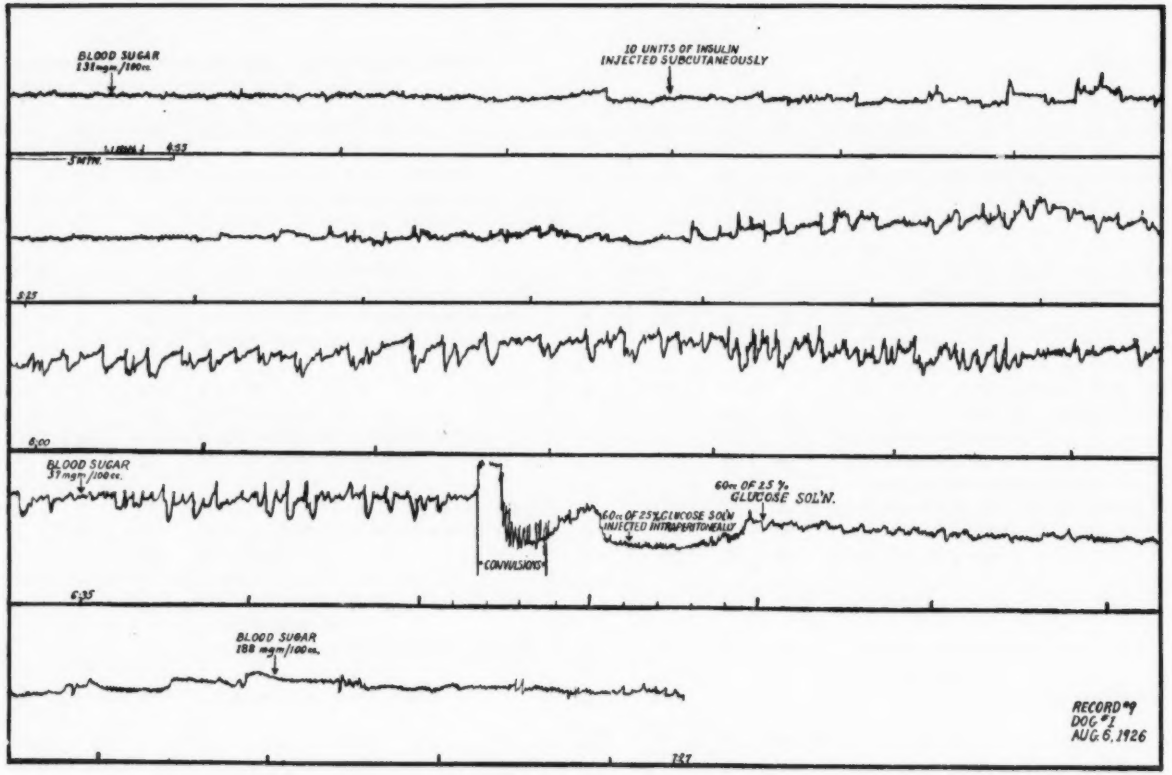

Fig. 1. This chart shows the effect of insulin on the gastric motility of a dog which had subsisted for 52 days on a diet deficient in vitamin B (see table 1, experiment 2). A gastric tracing taken on the 49th day of vitamin B deficiency showed only a few contractions during a period of six hours. On the 52nd day, the gastric record was begun at 2:28 p.m. and showed no contractions from 2:28 p.m. to 4:53 p.m. At 4:53 p.m. blood was drawn for sugar estimation (see figure). Insulin was then injected (2 units per kilo of body weight) at 5:10 p.m. This was followed by a marked rise in tonus and contractions which persisted until 6:47 p.m. when the animal developed convulsions which lasted about two minutes. A sterile solution of glucose was administered parenterally and the tracing continued until it resembled the pre-insulin record.

were likewise obtained in the case of dog 6 (experiment 13). The details of this experiment are presented in protocol 31 below.

PROTOCOL 31. Experiment 13. Dog 6. January 7, 1927,—76th day of vitamin B deficiency. Animal had shown partial loss of appetite for 10 days. A gastric record taken on the preceding day showed only slight rhythmic tonus changes throughout but no definite contractions during a period of 8.5 hours.

11:08–3:15.	Slight tonus variations; no contractions.
3:15.	Blood sugar: 121 mgm. per 100 cc. Hemoglobin: 68 per cent (normal hemoglobin was 98 per cent).
3:20.	Insulin (2 units per kilo) injected subcutaneously.
3:40–4:00.	Very small contractions and rise in tonus.
4:07–5:06.	Higher gastric tonus and definite contractions.
4:58.	Blood sugar: 63 mgm. per 100 cc.
5:06.	50 cc. of 15 per cent glucose solution injected subcutaneously.
5:15.	40 cc. of 15 per cent glucose solution injected subcutaneously.
5:06–6:00.	Contractions continue, but gastric tonus is lower.
6:09.	Blood sugar: 122 mgm. per 100 cc.
6:09–6:30.	Gastric tonus remains low; small contractions.
6:30–6:47.	Vigorous contractions.
6:47.	Blood sugar: 117 mgm. per 100 cc.
6:47–8:16.	Irregular small contractions; gastric tonus lower.
8:15.	Blood sugar: 74 mgm. per 100 cc.
8:16.	Record discontinued.

A study of the records of dog 6, taken after vitamin B therapy, is interesting. Thus, three tracings failed to reveal hunger contractions; nevertheless, when insulin was injected (protocol 33) during the time when the third tracing was taken, a definite response was obtained.

PROTOCOL 33. Experiment 15. Dog 6. January 13, 1927,—4th day after vitamin B therapy was begun. Animal slightly improved but gait still uncertain. Given large doses of vitavose during previous three days. Has eaten all food offered and increased from 8.9 to 10.1 kilos in body weight. Gastric records taken on January 10th and January 12th (over 2 and 3 hours respectively) showed no contractions.

9:00–10:42.	Complete quiescence.
10:42.	Blood sugar: 115 mgm. per 100 cc. Hemoglobin: 65 per cent.
10:54.	Insulin (2 units per kilo) injected subcutaneously.
10:54–11:45.	Practically no change in tracing.
11:45–12:10.	Much higher gastric tonus and definite contractions.
12:10.	Blood sugar: 52 mgm. per 100 cc. Hemoglobin: 65 per cent.
12:10–12:34.	50 cc. of 15 per cent glucose solution injected subcutaneously.
12:43.	50 cc. of 15 per cent glucose solution injected subcutaneously.
12:43–1:10.	Contractions continue.
1:10–1:50.	Gastric tonus lower and similar to that of pre-insulin record; small, irregular contractions.
1:50.	Blood sugar: 72 mgm. per 100 cc. Hemoglobin: 62 per cent.
2:20–2:44.	Vigorous contractions.
2:44–3:07.	Small, irregular contractions; gastric tonus remains low.
3:07–3:14.	Quiescent and similar to pre-insulin record.
3:14.	Blood sugar: 104 mgm. per 100 cc. Record discontinued.

The results of sixteen experiments in which insulin was administered parenterally to dogs in various stages of vitamin B deficiency may be briefly summarized as follows:

In four experiments, the injection of insulin was followed by an increase in gastric motility; in the case of one dog, however, vitamin B therapy had been instituted four days previous to the administration of insulin but normal hunger contractions had not reappeared.

In twelve other experiments, the injection of insulin failed to produce any significant change in the motility of the empty stomach.

DISCUSSION. In general a decrease in gastric motility was obtained when dogs were fed a diet complete except for vitamin B. However, two dogs manifested abnormally vigorous and long-maintained hunger contractions, lasting about seven hours continuously. The fistulae leaked considerably in both dogs. One of these dogs had severe anemia; the other manifested skin tenderness and was rather inactive. It is impossible to say whether these findings bear any relationship to the unusual gastric activity.

Gastric records taken on dog 4 showed a marked decrease in motility during vitamin B deficiency. On the 58th day insulin was injected (protocol 23, experiment 5) but no apparent effect was observed. Another insulin injection (experiment 7) was made on the 60th day of vitamin B deficiency and still no effect on gastric motility was observed. However, on the 62nd day, without any further administration of insulin vigorous hunger contractions, continuous over a period of three hours, were obtained. No interpretation can be placed on this result at present. A biologic assay of insulin (Stucky, 1928) failed to reveal the presence of an appreciable amount of vitamin B.

The disturbances in gastric motility observed in these vitamin B deficient animals do not appear to be related to the presence of hyperglycemia, as will be seen from inspection of table 1. Furthermore, in the majority of experiments, the hypoglycemia, which resulted from insulin injection, did not augment gastric motility. For this reason, one can not conclude that the few positive effects obtained are attributable solely to the effect of insulin on the blood sugar level.

A study of protocols[9] 32, 33 and 34 demonstrates that the doses of insulin employed in these experiments were without significant effect on blood concentration, although Drabkin (1925) and others have shown that massive doses (20 units per kilo) of insulin produce anhydremia in normal dogs.

[9] Protocols 32 and 34 may be found in the dissertation of C. J. Stucky (Yale University, 1927).

PROTOCOL 23. Experiment 5. Dog 4. August 12, 1926,—58th day of vitamin B deficiency; animal had shown an erratic appetite for over six weeks and lost 1.3 kilos (approximately 12 per cent) in body weight; otherwise the animal appeared to be quite normal; a gastric record taken 9 days before showed good contractions.

9:45 a.m.	Gastric tracing begun.
9:45–1:30	Quiescence.
1:30–2:00.	Record interrupted.
2:10–3:55.	Quiescence.
3:55.	Blood sugar: 93 mgm. per 100 cc.
3:59.	0.5 unit of insulin per kilo of body weight injected subcutaneously.
5:42.	Blood sugar: 57 mgm. per 100 cc.
5:52.	Tracing discontinued.
5:55.	10 gm. glucose (10 per cent solution) administered per os; dog very weak; unable to walk.
6:30.	Animal slightly better; hesitating gait.
6:40.	Blood sugar: 109 mgm. per 100 cc.
6:45	Insulin experiment discontinued.
9:30	No apparent symptoms.

SUMMARY AND CONCLUSIONS

Tracings of the gastric motility of seven dogs with simple gastric fistulae were taken at frequent intervals during the progress of and recovery from B-avitaminosis. One of these dogs was used in two series of experiments. Estimations of the blood sugar level were made during the course of these studies. In the majority of animals, a decrease in gastric motility developed. Insulin was then injected and the effects on the gastric tracing and the blood sugar level determined.

In the eight cases studied, six revealed a progressive decrease in gastric motility while two manifested abnormally vigorous and prolonged contractions during the progress of vitamin B deficiency.

Of sixteen insulin experiments performed on dogs showing a decrease in gastric motility, 25 per cent yielded what appeared to be a positive response to the hormone as indicated by a definite improvement in the motility of the stomach.

Since commercial insulin does not contain any appreciable amount of vitamin B, the favorable response obtained in a few cases can not be attributed to the presence of this factor in the hormone preparation employed.

No direct relationship between the blood sugar level and the gastric motility could be demonstrated under the conditions of these experiments.

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THE DIRECTION OF THE CILIARY CURRENTS IN THE OVIDUCTS OF VERTEBRATES

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It is commonly believed that the cilia of the oviducts in vertebrates produce currents running from the internal end of the duct toward its external opening. Even where the oviducts are highly specialized, as in mammals, this condition is supposed to prevail. Thus Piersol (1911) in his text-book on *Human Anatomy* remarks of the Fallopian tubes, that they are provided with cilia that produce a current directed from the infundibulum to the uterus and thus while facilitating the progress of the ova along the tube, the cilia retard the ascent of the spermatozoa. In the uterus also, according to this author, the cilia beat downward, that is, toward the external mouth. Thus even in the human species the course of the ciliary current in the oviducts is believed to be outward.

No one can have examined this current in a living oviduct without having been impressed by its vigor. In fact so strong is it that I have never been able to convince myself that spermatozoa could make headway against it, and yet these cells must, by one means or another, reach the proximal end of the oviduct in the neighborhood of which the egg is fertilized. As material for the study of these currents the oviduct of the turtle seemed to me a favorable object, and I therefore began an investigation of this general question on the oviducts of the common painted turtle, Chrysemys picta (Schneider).

In mature females of this species, whose carapace may measure 12 to 13 cm. in length, the oviduct is commonly about 15 cm. long. The proximal two-thirds of the duct are light-colored, soft-walled, and of relatively uniform diameter. It is usually collapsed, more or less transversely folded, and presents the appearance of a thick ribbon some 6 or 8 mm. in width. At its proximal end, next the ovary, this portion opens out into a wide infundibulum and at its distal end it constricts slightly as it passes over into the distal third. Internally the proximal portion is longitudinally corrugated, and, judging from the condition of the eggs occasionally found in it, its function is to contribute the small amount of albumen that surrounds the yolk of the turtle's egg.

The distal third of the oviduct is relatively thick-walled and resistant.

It is more or less darkly pigmented and in the majority of turtles that I examined (early summer) it contained three oval eggs end to end and ready to be laid. It is concerned with the secretion of the egg-shell and in freshly opened females its walls can be seen in local action slipping back and forth over the surfaces of the contained eggs, an activity dependent upon its musculature. Except where it is distended by an egg its diameter is a millimeter or so more than that of the proximal part. When freed of eggs its inner surface is marked by deep longitudinal corrugations that take a somewhat wavy course.

Observations on the cilia of the oviducts are best carried out on ducts devoid of eggs. Such ducts were dissected out of a turtle, thoroughly irrigated with Ringer's solution, and laid open from end to end by a single longitudinal incision. For the inspection of the ciliary currents transverse pieces of such a duct were cut out, folded under on what was their longitudinal axis and the folded edge was inspected through the microscope. The active cilia and a strong longitudinal current directed down the tube could be easily demonstrated. This current was seen in both the distal and the proximal portions of the tube.

In consequence of the greater smoothness of the inner surface in the proximal portion as compared with the corresponding surface of the distal portion most of the observations on the cilia of the oviducts were made on the proximal part. The currents in this portion when viewed under the microscope gave the impression of unusual strength and small particles were carried along in the adjacent fluid at such a rate as to lead one to believe, as already intimated, that no spermatozoön could make its way against the onrush. To ascertain what would happen to spermatozoa under such circumstances fresh sperm cells from the epididymis of a male turtle were discharged over a fold of living ciliated oviduct and their fate was watched under the microscope. Notwithstanding their own movements they were swept bodily down the oviduct. Not a single spermatozoön was seen to make any headway against the ciliary tide. This experiment was many times repeated and always with the same result. I therefore conclude that the literary efforts that have been expended by some authors in describing the rheotactic struggles of ambitious sperm in their attempts to reach and fertilize the egg at the top of the duct and thereby to demonstrate a certain kind of fitness for survival, find no justification in the turtle.

If spermatozoa through their own activity are unable to compete with the ciliary currents of the oviduct, by what means do they reach the upper end of this duct? Recent work on ciliary reversal (Twitty, 1928; Parker and Marks, 1928; Parker, 1928) led me to suspect that the cilia of the oviduct might be made to reverse their stroke temporarily by secretions contributed by the male to the spermatic fluid and thereby to carry the

sperm cells up instead of down the oviduct. I therefore prepared extracts of the turtle's testis, epididymis, vas deferens, and cloacal wall and applied these extracts separately and in combinations to the oviducal membranes, but in no instance was there the least evidence of reversal. The extract from the epididymis seemed at times to reduce the vigor of the ciliary stroke but otherwise no effect was observed. It appeared to me possible that what occurred was an inhibition of tubal ciliary action as a result of contact with the spermatic fluid, after which the sperm cells would be free to move of themselves throughout the length of the duct. But under the best of circumstances I never succeeded in slowing down the tubal currents to a point that warranted the assumption that the sperm could have made headway against these currents.

I therefore turned to a complete and detailed inspection of the inner surface of the oviduct to discover, if possible, the means of ascent for the spermatozoa. For reasons already given, I employed in this work the proximal rather than the distal portion of the duct. In this inspection I cut out a section of the duct, split it longitudinally and spread it out on a glass slide so that its inner face was exposed to view. By starting at one of its longitudinal edges the piece could be inspected under the microscope for the direction of its ciliary currents and rolled over on itself millimeter by millimeter till its whole transverse extent had been explored. On carrying out this examination I found that the cilia of almost the whole circumference of the tube beat distally but that there was in addition to this general field of downward activity, a narrow band of cilia, a couple of millimeters in width, in which the stroke was persistently and vigorously proximal in direction. It was possible so to fold a piece of the oviduct that on one edge the general ciliary current could be seen running distally and on the opposite edge the other current running proximally. From these observations I conclude that the oviducts of the turtle possess a double system of cilia, a broad general tract that covers most of the interior of the tube and whose direction of stroke is distal or toward the exterior, and a narrow restricted tract only a couple of millimeters in width whose direction of stroke is proximal or toward the interior. The first of these tracts in consequence of the direction of its stroke may be called the abovarian tract, the second the proövarian tract. The proövarian tract can be traced the whole length of the oviduct though it is less evident in the lower third of that organ than in the upper two thirds. Presumably it is this narrow proövarian tract that is concerned with the transportation of the spermatozoa from the region of their deposition by the male near the outer end of the oviduct to the region of their effectiveness near the ovary at the inner end of that tube.

The location of the proövarian tract in the oviduct of the turtle is clearly marked on the exterior of that organ. If the flattened proximal portion of

the turtle's oviduct be examined *in situ*, the two borders will be found to be quite different. One appears to be nothing more than the natural edge of the tube; the other is accompanied by blood vessels of considerable size. It is this vascular edge of the duct that marks on the exterior of the organ the position of the internal band of proövarian cilia, a band which comes thus to lie in what is almost a groove on the inner face of the oviduct. As this vascular edge in its course over the proximal portion of the oviduct approaches the distal portion of that tube the edge assumes more and more a blackish color and on the distal third of the duct, it can be traced as a black streak over the whole length of this portion.

Judging from these various observations it seems probable that the spermatozoa of the turtle pass up the oviducts of this animal not by their own activity, nor by ciliary reversal, nor by the muscular movements of the ducts themselves, but by the action of a special, narrow tract of cilia, the proövarian tract, whose beat is persistently and vigorously toward the ovary. The remainder of the oviduct wall is covered with abovarian cilia whose beat toward the exterior keeps the tubes clear and possibly accomplishes in part the outward transportation of the eggs. Whether such a double ciliary system as this obtains in the oviducts of other reptiles than turtles, in those of birds or of mammals remains to be ascertained.

SUMMARY

1. The oviducts of the turtle, Chrysemys picta (Schneider), possess two systems of cilia; a general system covering most of the interior of the duct and beating away from the ovary (abovarian system) and a restricted narrow tract of cilia extending the length of the duct and beating toward the ovary (proövarian system).

2. Spermatozoa reach the ovarian end of the oviduct not by their own activity, nor by the muscular movement of the oviduct, nor by the reversal of the ciliary beat in this duct, but by transportation afforded by the proovarian ciliary tract.

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STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN BIRDS

XXV. The Action of the Ovarian and Placental Hormone in the Pigeon

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Some of the recent work on the ovarian and placental hormone (follicular, female, menformon) has emphasized or suggested the capacity of this hormone to hasten the appearance of puberty or sexual maturity. The present study was made with special reference to a test of this point. The earliest age at which sexual maturity (as indicated by the production of the first egg) may be expected in our ring doves has been determined by numerous complete records kept during many years. This material therefore seemed specially favorable for this test. Again, when our studies were made—July, 1925 to July, 1926—the action of this hormone had been tested in mammals only and it was obviously necessary to test its action in other animal groups.

The successful application by Allen and Doisy of the vaginal smear method for the detection in mammals of the activity of an extract has served well to multiply studies on this hormone; but, a resulting relative inattention to weight changes in germ glands, has probably led in some cases to a misconception of the capacity of this hormone to hasten the appearance of sexual maturity. The data presented here show adverse, not favorable, effects on ovarian development and growth. It has also seemed equally important to learn the action of this hormone on the testes of young males, since it is becoming increasingly probable that fetus and embryo of mammal and bird are subjected to its action—with important results on some aspects of prenatal growth. We have therefore investigated this subject and find that this incretion also has adverse effects on the growth of the testes. The ovarian and placental hormone unquestionably induces growth in the uterus and mammary of mammals, and less readily in the oviduct of birds—but it does not induce sexual maturity. Under normal conditions this hormone is not the *cause* but a *result*, of a sexually mature gonad.

While this study was in progress we made a report (Riddle and Tange, 1926) of the results obtained from the first seven immature females treated, and on the failure of the hormone to modify ovulation rate or sex behavior in adult females. These early results—dealing with females only—showed that even larger dosage than that used in mammals, often or usually failed to elicit a clear response in birds; these results were therefore entitled "Some limitations of the action of the follicular hormone in birds." Among the seven immature females treated it was found that probable growth and unquestioned hyperemia of the oviduct could be declared in only three cases though relatively high dosage was administered. These few cases however, did serve to show that the typical action of this hormone—namely, the induction of a temporary uterine (oviducal) growth and hyperemia—was not limited to mammals.

METHODS AND MATERIAL. Five separate preparations of the hormone from the follicular fluid of the sow were used by us. Two of these were prepared by the Doisy and Allen (1924) method, and two by the shorter method of Ralls, Jordan and Doisy (1926). The raw fluid was supplied by Armour & Company. One of these preparations was tested and induced active growth in the uteri of young rabbits. One lot was kindly supplied and tested by Dr. Edgar Allen. Three tested preparations of the placental hormone were contributed by Drs. R. T. Frank and R. G. Gustavson. All of our own preparations were made in the shortest possible time, and the injections began at once. The preparations first used were dissolved in oil, but since this was not well tolerated by our animals the later ones were dissolved in glycerin. Injections were subcutaneous, in breast and nape, except in the case of one male and three females (intraperitoneal), and made 3 times daily.

Tests were made on 27 immature female ring doves, and on a number of mature females for purposes which will be noted later. Adequate tests were made on 16 immature males. Since there is very considerable variation in the size of the normal oviduct and of the gonads in different immature birds of the same age, it is clear that the matter of adequate "controls" is of much importance. We have sought to meet this need, first by supplying two or three controls for each treated animal; and second, by providing a number of age-groups, each of which is of some further service as controls for preceding and succeeding age-groups. Only about half of our total of control birds were killed and examined at the same time as the treated birds. During the immediately following year it was necessary to obtain precisely similar control for a study on the effects of the hormone of the anterior pituitary (Riddle and Flemion, 1928). In order better to show the normal size variation of the glands in these ring doves the controls taken for the two separate studies have now been combined for use in both of these studies.

PRESENTATION OF DATA. *Effects on the ovary of immature birds.* A summary of data for ovarian and oviducal size obtained on immature females aged 2.2 to 4.4 months is given in table 1. Here only average, maximum and minimum size within an age-group is given; otherwise the tabulation would require very much space. It will be observed that the ovaries of all of the 8 groups of treated birds average smaller than their

TABLE 1

Weights of ovaries and oviducts from immature female ring doves injected with ovarian or placental hormone—and their controls

GROUP	CONTROL OR TREATED	NUMBER	AGE	WEIGHT OF OVARY			WEIGHT OF OVIDUCT		
				Average	Maximum	Minimum	Average	Maximum	Minimum
			months	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	Control	7	2.2	15.3	24.3	7.3	17.2	30.3	12.5
	Control	4	2.4	16.1	18.6	14.1	17.9	28.5	10.0
	Treated	2	2.3	15.5	20.5	10.5	21.9	30.6	13.2
2	Control	4	2.6	13.7	18.8	10.0	15.0	19.4	11.0
	Treated	2	2.6	11.3	11.8	10.7	24.9	35.5*	14.2
3	Control	8	2.8	27.4	60.0	11.0	19.5	24.3	15.0
	Treated	3	2.9	18.0	25.2	14.2	23.2	28.8	20.2
4	Control	8	3.1	26.3	52.0	12.9	27.4	36.7	19.0
	Treated	4	3.2	11.4	14.7	8.0	22.1	38.0*	10.6
5	Control	4	3.5	24.5	27.6	18.4	21.3	26.8	15.8
	Treated	3	3.4	12.9	17.0	9.4	14.9	19.5	11.5
6	Control	5	3.7	39.9	60.9	28.1	70.2	211.0	20.2
	Treated	5	3.7	27.0	34.6	20.8	59.4	170.7*	17.2
7	Control	13	4.0	68.8	353.0	30.0	51.4	235.0	15.3
	Treated	3	4.1	22.7	36.1	7.2	58.0	129.2*	15.5
8	Control	17	4.5	50.8	110.1	20.9	47.2	177.0	16.6
	Treated	3	4.4	22.5	29.2	12.0	74.1	109.8*	26.5

* Hormone obtained from placenta used here.

controls—the difference ranging from 1.3 to 67 per cent, with an average group reduction of 39 per cent for the 8 groups. In no case does the maximum size of a treated individual equal the maximum weight of its control. There can be no doubt that the material injected into these birds not only checked the growth of their ovaries, but it also led to an actual reduction in the size of their ovaries.

This reduction in size of the ovaries, as shown by comparison of weights

of treated and control, was confirmed by the appearance of the larger follicles in the older (immature) ovaries. In the bird's ovary the individual ova soon become observable macroscopically. In most cases these larger follicles showed clear signs of *degeneration*, and this was confirmed by the histological examination of three typical cases.

Effects on the oviduct of immature birds. The data obtained on size of oviduct in treated and control show a different, though a mixed, situation. In 5 groups the treated oviducts average larger, in 3 groups they average smaller, than their controls. Considered as groups the oviducts of treated birds average nearly 15 per cent larger than their control.

The real evidence for a stimulating effect of the hormone on some of the oviducts rests, however, on a closer examination of the individual data. For example, one preparation of placental hormone was injected into 4 immature females and in every case the oviduct of the treated bird was larger than the average control (38.0: 27.4; 170.7:70.2; 129.2:51.4 or 70.2; 109.8:47.2 or 70.2 mgm.). In the first of these cases the bird was aged 3.0 months and its oviduct was larger than the largest of the 8 control of slightly older age (3.1 months). This case must be considered a positive result, and the preparation used gave additional evidence of its potency. Another placental extract was injected into two immature females. One, aged 3.7 months, had an ovary smaller than those of its 5 control (29.7: 39.9 mgm.), but its oviduct was much larger (170.7:70.2). The other test was made in an older bird (6.4 months; not listed in table 1) whose ovary was scarcely the equivalent of that of its 6 control (92.0:93.2 mgm.), but its oviduct was evidently enlarged as a result of treatment (286.0:82.9; largest of control, 187.0 mgm.). A third placental extract gave less uniform evidence of a capacity to induce oviducal growth. Nine tests with larger or smaller dosage were made with this preparation. In none of 4 tests with the smaller dosage (presumably 8 rat units during 3 to 4 days) did the weight of an oviduct equal those of the controls; in 3 of the 5 tests with larger and longer dosage (15 rat units in 7 days) the treated oviducts were larger than the average control; in the case of only one bird (2.5 months) is it evident that oviducal growth (35.5 mgm.) resulted from the treatment. Obvious hyperemia of the oviduct was found in each of the few cases in which a positive growth effect was found, and also in 2 additional cases treated with hormone of placental origin.

Twelve immature females were injected with hormone prepared from the follicular fluid of the sow. The dosage probably varied from 5 to 22 rat units. In 11 of these cases the size of the treated ovary was smaller than that of its control. Hyperemia and hyperplasia of the oviduct were clearly shown in only a single test. This was found in the youngest bird (2.2 months) treated, and in a bird given the maximum dose (22 units) in 3 days, followed by an interval of two days preceding autopsy. Its

oviduct was larger than the largest of 9 (or 21) control—being 78 per cent larger than the average of the 7 birds strictly of its own age. In only 2 other cases were the oviducts of birds treated with the follicular hormone larger than the average of their control, and in neither case did they exceed

TABLE 2

Weights of testes from immature male ring doves injected with ovarian or placental hormone—and their controls

GROUP	CONTROL OR TREATED	NUMBER	AGE	TESTIS WEIGHT			BODY WEIGHT	
				Average	Maximum	Minimum	Start	End
			months	*mgm.*	*mgm.*	*mgm.*	*grams*	*grams*
1	Control	4	2.2	6.9	8.5	4.9		151
	Treated	A	2.2	9.0			134	125
2	Control	6	2.4	6.5	8.3	4.2		149
	Treated	B	2.4	6.8*			148	147
		C	2.6	7.2			146	136
3	Control	13	3.1	8.6	16.8	3.9		152
	Treated	D	3.1	5.3*			139	149
		E	3.2	8.1			141	134
		F	3.2	8.8			119	121
		G	3.2	6.5			127	119
4	Control	7	3.4	8.4	13.0	5.3		153
	Treated	H	3.4	12.0*			143	147
		I	3.5	5.6*			132	132
5	Control	9	3.8	29.2	102.6	5.7		145
	Treated	J	3.6	11.4			144	142
		K	3.8	8.3*			141	137
		L	3.8	11.3			159	154
		M	3.9	13.7			144	136
		N	3.9	9.3			148	137
6	Control	5	4.5	20.1	34.3	14.2		146
	Treated	P	4.5	9.2*			162	158
7	Control	3	4.7	85.6	210.0	19.2		151
	Treated	Q	4.7	36.4			158	145

* Hormone obtained from placenta used here.

the largest of their control. Certainly in 8, and probably in 11, of the 12 tests the hormone obtained from follicular fluid failed to produce measurable growth in the oviducts of these birds. These results like those obtained with the hormone derived from the placenta, confirm our earlier statement (1926) that "our results indicate a less ready, and a less varied, response of the bird to this substance than has been found for mammals."

Effects on testes of immature males. Six young males were treated during 3 to 7 days with 5 to 15 rat units of hormone derived from placenta. Ten others were each given during 3 to 7 days the hormone obtained from 14 to 28 cc. of crude follicular fluid (two of these each received 20 rat units in 4 days). The results are fully given in table 2. On the average we find that the males were somewhat less heavily dosed than were the females.

In general it will be seen that the treatment resulted in a reduction of testis size. This is best shown in those age-groups (3 and 5) with the largest number of treated and of control for comparison. Thus the testes of 4 treated birds in group 3 average less than 84 per cent of the size of their 13 controls. The testes of 5 treated birds of group 5 average only 37 per cent of the testis size of their 9 controls. The single bird (A) treated at 2.2 months had slightly larger testes than the data for control would lead

TABLE 3

Average weights of certain organs of all immature (2.2 to 6.4 months) treated doves—and their controls

SEX	CONTROL OR TREATED	NUMBER	WEIGHT OF:						
			Thyroids	Adrenals	Liver	Spleen	Bursa fabricii	Body	
								Start	End
			mgm.	*mgm.*	*grams*	*mgm.*	*mgm.*	*grams*	*grams*
Females	Control	82	13.9	10.6	3.153	69	164		139
	Treated	27	17.5	10.9	3.219	85	115	137	133
Males*	Control	60	14.5	9.8	3.198	81	197		149
	Treated	16	14.8	10.5	3.314	106	109	143	139

* Unequal numbers of males and females taken at any one stage of immaturity make any comparison of the two sexes here invalid.

us to expect. This bird, however, received the minimum dosage, and this during a period of only 3 days. It is also to be observed that though most of the birds suffered a slight reduction in body weight during the period of injection this did not occur in 4 of the 16 cases.

Size of organs other than genital organs. Possible effects of the ovarian and placental hormone on size changes in other than genital structures is a question which should ultimately be resolved by studies of the kind described here. The data of table 3 are submitted with some notable reservations. Only a very full analysis and presentation of these data could give a really true picture of the results. We present, however, only the shortest possible summary.

Within the age limits of the various birds included in table 3 it is found that body weights, and possibly thyroid weights, increase with age; the liver and spleen probably decrease with age; the bursa Fabricii in-

creases to 3.1 months and decreases thereafter; any change in the suprarenals is uncertain. Since the proportion of total treated and control taken at younger and older ages varies considerably in the female (table 1) and male (table 2) series, it is clear that our summary can not afford a proper comparison of the size of any of these organs in the two sexes. Similar inequalities are introduced to a less extent between our summarized values of treated and control of the same sex. Adequate analysis of the data shows, however, that the spleens of treated birds were consistently enlarged as the result of treatment; though it is equally important to note that our experience indicates that such enlargement attends the injection of very many substances, and this enlargement is therefore in no way a specific response to this hormone. The bursa is also markedly and consistently decreased; but this too attends *all* influences adverse to the bird's health and growth. That the injections resulted, on the whole, in a decrease of body weight is properly shown in this summary. The variation of thyroid size in our material is very great; it is very questionable whether the apparent differences in weight of control and treated thyroids, suprarenals and livers are really significant. When other work shall have supplied similar data on a considerable variety of animal species this question can probably be resolved by an examination of the total of the data then available. In this table are included two treated immature females of ages too advanced (5.1 and 6.4 months) to include in table 1; the controls for these two birds are also included here.

The effect of blank injections of glycerin only. Since we early noted an adverse reaction of the skin and tissues of the bird to injection of the hormone dissolved in oil, we utilized a glycerin solution in most of our tests. This solution also plainly produced some irritation and it was thought advisable to inject some of our control birds with glycerin only. Twelve immature birds—4 males and 8 females—were given intraperitoneally 0.3 cc. of glycerin daily during 10 days. This treatment was rather more prolonged than that given to the hormone injected birds (3 to 7 days). We wished, however, to obtain a fair picture of the changes which would accompany the injection of glycerin alone. These birds therefore may be spoken of as "injected controls."

The results may be stated as follows: The average body weight of males decreased from 144 to 139 grams; that of females from 132 to 126. The testes of the 4 males averaged 6.2 per cent heavier than uninjected controls. The ovaries of the 8 females averaged 12.4 per cent lower than uninjected controls; their oviducts were 3.2 per cent heavier. Every bursa Fabricii was smaller than its control. Every thyroid was smaller than its uninjected control (all were killed at a season of small thyroid size—September —while other controls were killed throughout the year). Every spleen was *smaller* than the average of its uninjected control. In 7 cases the liver

was smaller, and in 5 cases larger, in the injected control. It is therefore probable that the adverse effect of our hormone preparations on body weight may be ascribed to the solute—glycerin; it is clear that the size changes found in testis, ovary, oviduct, and spleen are not ascribable to the glycerin used.

Effects on age at maturity, and on mature females. In an earlier paper (Riddle and Tange, 1926) we considered the failure of the hormone to cause measurable increase in the rate at which eggs were formed in the adult ovary. This was tested in 6 cases where egg-laying was not normally frequent. Of somewhat similar nature are two additional tests in which we sought to learn whether the hormone injected into "incubating" females would cause them to stop incubation, or nesting behavior, and perhaps produce additional eggs while still nesting. Both birds deserted their nests—after 9 and 11 days of incubation—but we are in doubt whether the hormone or the disturbance incidental to 3 injections daily was the more responsible. Certainly new ova did not begin their final period of rapid growth until *after* incubation was discontinued. This was proved by the time of laying of their next eggs.

We have also made an attempt to learn whether birds injected at an early immature age would produce their first egg—would become in fact sexually mature—at an abnormally early age. For this purpose we injected each of 4 birds, aged 2.2 to 2.8 months, with approximately 25 rat units of hormone from the follicle during 7 days. They were then kept in pens containing many doves of approximately their own age. From the data of Riddle (1928) it is clear that very rarely (3 of 977 cases) these doves may mature without treatment at so young an age as 4.0 months, though the greatest number mature at about 7.5 to 8.0 months. One of the 4 birds used in this test died of an acute infection, while still immature, at 7.2 months. The other 3 birds produced first eggs at 5.5, 5.6 and 7.6 months. These cases indicate that the administration of the ovarian hormone does not hasten the attainment of sexual maturity.

Discussion. The potency and action of extracts of ovary and placenta have been studied hitherto chiefly by two methods. First, by a period of dosage followed by an examination of the changes in size or weight of certain organs—notably the uterus. Second, the application of the "vaginal smear" method of Stockard and Papanicolaou (1917) in combination with improved methods of hormone preparation more recently accomplished by Allen and Doisy (1923). This method has proved invaluable for standardizing extracts, and for testing the potency of extracts in mammals. In other animals the vaginal smear method is as yet of little or no importance. Even in mammals the further employment of this method is not at all adapted to testing the action of the hormone on the several organs of the body other than the uterus. For this important purpose the first method is indispensable.

The present study was largely concerned with size changes in organs—particularly in the ovary and testis. On these two organs the injections have been found by us to produce inhibition of growth, and doubtless also a definite decrease in size. Other organs were also examined by us, but in some of these the variations in size (in both treated and control), and a lack of consistent differences under treatment, make a conclusion on the basis of our data alone quite impossible. When we attempt to place our data on change of size in organs alongside the results of others we find some contradiction and much confusion. Much of this may be ascribed to the fact that the earliest work employed preparations of this hormone which were even less pure than those employed during the last 4 years. But no careful review of the available data can fail to note that the number and quality of the control animals (and treated) do not justify many of the conclusions that have been drawn. Again, in some cases actual weights and measurements have been so scantily published as to be without value for other or further investigation.

This hormone, or extracts almost certainly containing it, has been reported to have an antagonistic action on the testes in the following cases: Rats, rabbits and guinea pigs—except the very young of the latter (Herrmann and Stein, 1920); rabbits (Fellner, 1921; Glimm and Wadehn, 1926); rats (Laqueur, Hart and de Jongh, 1926; Steinach and Kun, 1926). Bugbee and Simond (1926), however, report "no antimasculinizing effect" in rats. We note that they made no weighings of testes; and that, in their special test of this point, 3 rats treated seem to have fertilized ova a little later than their control. Their injections did not hasten sexual maturity in either male or female rats. Our own data clearly show that the preparations used by us are antagonistic to the growth of the testes of doves.

It is important to observe that the injection of this hormone in postnatal stages have given fairly uniform repression of growth on the accessory genitals of the male: Epididymis, prostate, penis of rabbits, rats and guinea pigs (Herrmann and Stein, 1920); epididymis, rabbits (Fellner, 1921); prostate, seminal vesicle, penis of rats (Steinach and Kun, 1926; Laqueur, Hart, de Jongh, 1926). On the other hand it is precisely on accessory organs of the female—uterus and mammary—where the hormone has its distinctly growth-promoting effects. In these organs therefore the hormone has given clear evidence of special adverse effects in males.

Surprisingly little has been observed on effects of this hormone on the size of the ovary. Brouha and Simonnet (1925) found an increased size of the rat uterus without increase in ovary. Zondek and Aschheim (1926) find that in mice folliculation is not induced, nor does premature ripening of ova occur; these, they later (1927; and Smith, 1926) point out, are effects of the hormone of the anterior hypophysis. In at least some of

their earlier papers, Allen and Doisy (1924), and Frank, Kingery and Gustavson (1925), have considered the ovarian hormone as the one which brings about puberty, or sexual maturity. This would seem necessarily to imply that the hormone favorably affects ovarian growth and development. Our own results clearly indicate that the hormone quite definitely opposes the growth, and even markedly reduces the size, of the immature ovary of the bird. The development of ovary and testis in these birds is accelerated by the hormone of the anterior pituitary—as shown by Riddle and Flemion (1928) in a paper published alongside the present study. It now seems clear that the supposed relation of the ovarian and placental hormone to sexual maturity must be entirely abandoned.

It is becoming evident that this ovarian hormone first appears normally, and in notable quantity, at or near puberty. The available evidence suggests that the hormone of the anterior pituitary also begins to appear in larger quantity—or at least to exert a greater effect on the gonads—at puberty. If we are to understand the marked changes which occur during adolescence it would therefore seem necessary to learn fully the separate action of these two hormones on the entire organism.

Some effects of this hormone on incretory organs other than testis and ovary have been reported. Iscovesco (1913) speaks for an increase of thyroid size in rabbits, and Fellner (1913) found the suprarenals usually enlarged—though he notes a high death rate from the injections. Ceresoli (1924) finds enlargement of both thyroids and suprarenals in guinea pigs. Suzuki (1925) according to an incomplete abstract, obtained an enlargement of the thyroid and atrophy of the thymus. We find that though the thymus and bursa Fabricii are definitely reduced in size by the treatment, this can in no sense be considered a specific effect of the hormone since they are similarly reduced by a wide variety of substances—and by practically any *condition* this is unfavorable for health and growth. The thyroids and adrenals of our treated birds yield averages not definitely different from those of the control—though they vary from the control in the same sense as in the studies cited above. Our birds show quite unusual differences of thyroid size. Only similar studies made on sufficient numbers of treated and control in other animal species can really decide whether such differences are significant, and a specific result of the treatment. The bird's spleen is definitely found to enlarge under this treatment; but so many substances do the same thing that it is very doubtful that it represents a specific action of the hormone. The changes found in the bird's liver are much less marked than those in the spleen. It is notable, however, that here—as elsewhere in our experience—the size changes in liver and spleen go in the same direction.

Whether in adult females the administration of this hormone results in any stimulation of the ovary to egg-production is not yet satisfactorily

determined. Steinach, Heinlein and Wiesner (1925) reported the reactivation of the senile ovary of rats. Slonaker (1927) thinks that after the menopause ovulations are not induced in the rat. Loeb and Kountz (1928) conclude that this hormone accelerates the maturation of follicles in the immature animals, but inhibits ovulation in guinea pigs. Riddle and Tange (1926) reported 6 failures and no successes in ring doves. Loewe, Voss and Paas (1927) think they obtained, from tests made on two adult fowls, an increased number of eggs as the result of such treatment. We think that tests of this particular question are better made on doves than on fowls, but admit that further tests are desirable. Terada (1926) noted adverse effects in the ovary of mature rats, but practically no effects on immature ovaries.

For these and further studies on the action of this hormone it is most unfortunate that the hormone is not available in a chemically pure form. At present, we can not be entirely sure that the various effects described here are in fact effects of the pure hormone. It seems also highly desirable that in further studies on size changes in organs the hormone should be dissolved in an entirely inert medium—inert toward all tissues and organs. These difficulties seem for the present to prevent a clear solution of the effect of this hormone on the development of the embryo. In mammals a considerable amount of evidence indicates that this hormone traverses the placenta (Courrier, 1924) and produces various distinct growth effects (Riddle, 1927) in the embryo. In the case of birds, Fellner (1925) has found the hormone in the egg-yolk, so that these embryos would seem necessarily to be subjected during the sensitive period of embryonic development to the action of this hormone. Riddle (1928) has found that at the time of hatching the testes of doves are only half as large as the ovaries, and is inclined to interpret these abnormal size relations as a result of the action of the ovarian hormone. That view would be supported by the finding, in postnatal stages, of adverse effects on testis growth and favorable or neutral effects on ovarian growth. Using such preparations of this hormone as are now available we have obtained adverse effects in both testis and ovary.

It thus results that growth phenomena in both mammalian and avian embryos suggest a differential action of this hormone on several organs of the male and female fetus. Experimental studies on postnatal stages of mammals have indicated that the accessory genital organs of the male are adversely affected by this hormone; growth in the accessory genitals of the female is influenced favorably. The present study on young postnatal stages of the bird indicates a similar adverse action on the primary germ glands of both sexes.

SUMMARY

The injection of the ovarian and placental hormone into immature doves does not hasten the attainment of sexual maturity in these animals. The idea that it does so in animals should be abandoned.

Effects of subcutaneous injections of this hormone, during 3 to 7 days, on various organ weights were studied in 27 immature females (82 controls) and on 16 immature males (60 controls). In this part of the study the animals were killed for examination within 6 to 72 hours after the last injection.

In both the ovaries and testes of these birds growth was stopped, and an actual reduction in size usually occurred, as a result of this treatment.

The oviducts of a few, but not all, of the treated birds showed the hyperplasia and hyperemia which characterize the action of this hormone.

Weights of body, thyroids, suprarenals, liver, spleen and bursa Fabricii were obtained in treated and control. The apparent changes in these organs are subject to qualifications fully described. The action of this hormone will remain very imperfectly known until this has been studied on many organs other than the uterus and mammary of mammals.

The true action of this hormone on the embryonic growth of certain organs in mammal and bird can be tested properly only when the hormone has been obtained in a pure form. At present the hormone appears to be favorable for growth in accessory female genital organs only; it is adverse to growth in accessory male genitals, and to growth in the primary germ glands of both sexes.

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STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN BIRDS

XXVI. The Rôle of the Anterior Pituitary in Hastening Sexual Maturity in Ring Doves

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Among those investigators who have attempted to isolate active principles from the anterior hypophysis there exists a widespread opinion that this organ produces more than one hormone. Newer studies throw much doubt on the activity of any of the truly specific substances of the anterior pituitary when these are merely fed to birds or mammals (Smith, 1927). On the other hand, the injection and transplantation of the fresh substance has recently given satisfactory proofs that this organ prepares one or more products of endocrine nature. By daily intraperitoneal injections of heavy doses of a particular extract Evans and Long (1922) were able to demonstrate the existence of a growth-promoting substance; though this particular extract, or the high dosage used, had adverse effects on growth in the ovary and testis. Another hormone, or at least another and different action of a substance contained in this gland, has since been found. Very recently, and quite independently, Smith (1926) and Zondek and Aschheim (1927) have shown that daily transplantation of pituitary substance effects a specific and highly favorable action on the growth of the ovary—inducing precocious sexual maturity in rats and mice. Smith (1927) soon extended these results to the male; and Smith and Engle (1927) have made notable contributions to many aspects of this problem.

The present study is concerned particularly with the anterior lobe as a source of the hormone which hastens sexual maturity, and incidentally with the necessary methods of extracting it and administering it. Riddle and Tange (1928), in a paper published alongside the present study, have shown that the hormone of the ovary and placenta does not hasten maturity in ring doves; when that hormone is artificially supplied it actually opposes the growth and development of the immature ovary, ovum and testis. Riddle (1928) has also supplied some evidence that normally in these same animals the bursa Fabricii and the thymus exercise a retarding action on the growth of the testes and ovaries, and thus exert a tendency to delay the time of sexual maturity. If the early results of Smith and of Zondek

and Aschheim (these were the only studies published at the time our work was done) could be demonstrated to hold also in ring doves we would here have a case in which the forces which hasten, and the forces which delay, the appearance of sexual maturity are both largely identified in one and the same animal.

The results of this study show that proper and adequate administration of anterior lobe substance to immature doves greatly hastens the growth of the testes and, to a lesser and very inconstant degree, that of the ovaries. Otherwise, our findings are of an inconstant and mixed nature—as are those from most of the few investigations hitherto made on this subject. It is fairly clear, however, that the anterior pituitary acts in birds as it does in mammals in the determination of age at sexual maturity. Daily pituitary homeotransplants during 9 to 25 days, from mature donors into immature doves, have proved less effective in causing gonad growth than the injection of a glycerin extract of the fresh gland. Small amounts of an acid extract, and of an alcohol extract of the acid treated residue, were probably without effect.

Results. Immature male and female doves received anterior pituitary tissue in three different forms: *a.* The fresh gland transplanted from mature male and female doves into breast muscle or (rarely) beneath the skin. *b.* Injection of a glycerin extract of fresh bovine glands. *c.* Injection of small amounts of acid or alcohol (heated) extracts of fresh bovine glands.

Transplantation tests. Altogether 509 anterior pituitaries were dissected from mature doves and pigeons and transplanted into the breast muscle of young doves. Transplantation was usually completed within less than two minutes of the death of the donor. Three of the young died after receiving a total of only 17 transplants and are not further considered here. From 9 to 25 transplants were made into each of the remaining birds. The amount and distribution of this dosage among the different birds, and the age of each bird at first and last dosage, are given in table 1. In a few cases very young birds were dosed for one week and were thereafter not again dosed until a period of 10 to 14 days before autopsy.

The effects of these transplants on size of testis and ovary are best judged by reference to figures 1 and 2. In these figures the gonad weights of treated young killed at various ages are indicated by small vertical bars placed on a horizontal scale. The control data are so placed on an adjacent scale as to permit a better judgment of the results of treatment than could be obtained from group averages or from tabulated data. On these graphs the gonads from birds which had received transplants are labelled "transplanted;" those from birds treated with extracts are labelled "injected."

The graphs show that nearly all of the 13 males treated by transplantation had larger testes than the comparable group of control would lead us

TABLE 1

Data concerning age, dosage and final body weight of immature doves receiving anterior pituitaries by transplantation

NUMBER	NUMBER TRANSPLANTS	AGE AT			BODY WEIGHT OF			
		Transplants		Autopsy	Treated		Control	
		First	Last		Start	End	End	Number
Males								
		days	*days*	*days*	*grams*	*grams*	*grams*	
1	18	103	121	123	148	149	149	6
2	20	60	118	121	147	143	149	6
3	9	109	119	121	141	143	149	6
4	19	35	116	120		135	149	6
5	20	38	118	120		149	149	6
6	13	100	117	118	159	143	145	9
7	20	52	113	118		143	145	9
8	20	33	115	118		131	145	9
9	20	54	115	117	145	147	145	9
10	19	25	112	117		148	145	9
11	20	31	114	116		125	145	9
12	9	58	76	90	128	143	152	13
13	11	61	73	74	151	138*	149	6
Females								
1	9	111	121	123	146	137	140	13
2	20	20	108	120		142	140	13
3	25	19	118	120		148	140	13
4	12	59	82	119	140	139	140	13
5	23	37	117	119		138	140	13
6	21	40	117	119		146	140	13
7	20	18	106	119		157	140	13
8	19	27	114	119		146	140	13
9	15	97	115	119	147	148	140	13
10	19	78	117	118	127	138	140	13
11	15	95	113	117	140	141	140	13
12	18	95	112	113	148	146	144	5
13	12	59	72	73	132	112*	134	4

* Died.

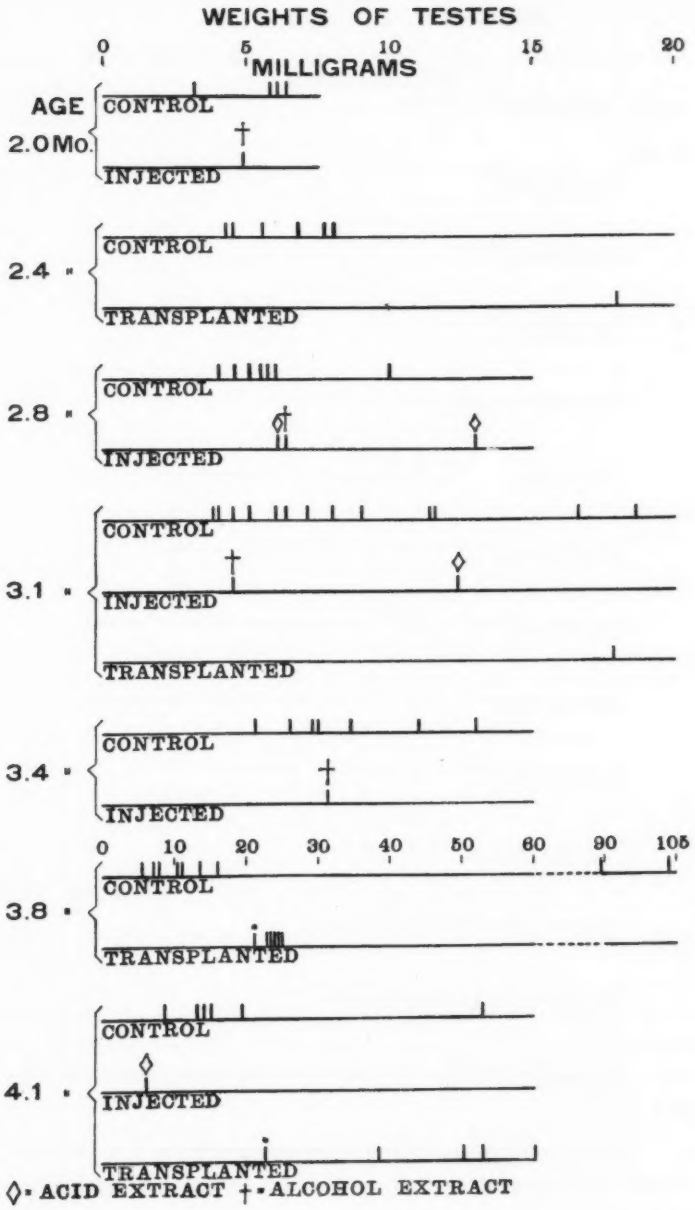

Fig. 1. Graphic presentation of testis weights from immature doves of various ages.

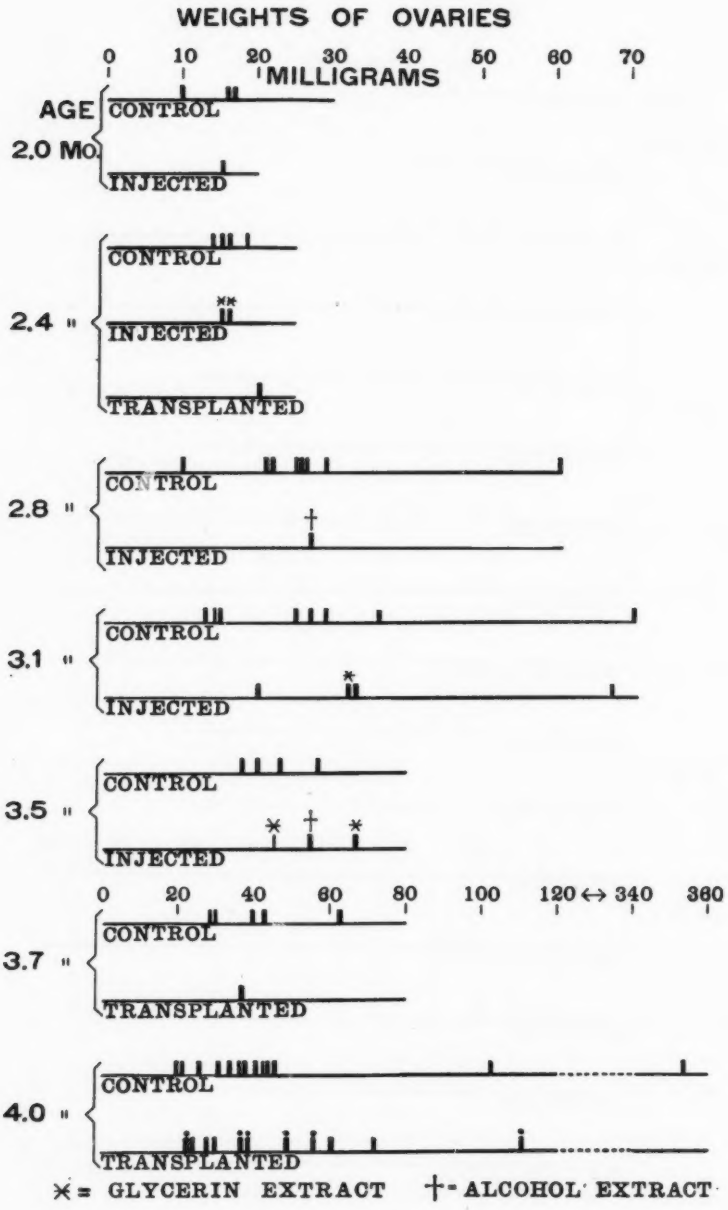

Fig. 2. Graphic presentation of weights of ovaries from immature doves of various ages.

to expect. It is practically impossible to state the amount of the increase in exact quantitative terms; but it is evident that some, and possibly all, of these testes were enlarged as a result of treatment. In the case of the 13 young females which received transplants the results, however, are much less evident. A few ovaries exceeded their expected weight, some were plainly unaffected, and others are indeed quite doubtful. Possibly some of this doubt arises from the fact that 11 of this group of 13 were killed at the age of 4.0 months—precisely the youngest age at which a normal dove may become sexually mature, and therefore the period of greatest variability in the normal size of the ovaries. It is nevertheless clear that, in our tests made with transplants, the growth of the gonads of female doves was accelerated less than were those of males.

One young female common pigeon received 20 transplants from adult common pigeons during a period of 41 days. When examined at the age of 4.0 months the ovary was found to be smaller (51 mgm.) than the ovaries of two of the three controls (average 76 mgm.)

Injection of a glycerin extract. A commercial (Lang, New York) glycerin extract of fresh unfrozen bovine glands was daily injected intraperitoneally into 11 males and 5 female immature doves. It was found that subcutaneous dosage was badly tolerated. The dosage was 0.1 to 0.3 cc. Three birds were killed and lost by a dosage of 0.5 cc. Dosage varied from 7 to 17 days, though no bird received more than 12 injections. In a few cases a 4-day intermission followed 7 days of treatment; 5 final injections were then made before autopsy.

In the case of the treated males the result on the growth of the testes is definite and striking. In 9 of the 11 tests the testes from treated birds are larger than the largest obtained from a much larger series of controls. The two remaining cases are from three tests made with the smallest dosage; they are nearly double the size of the average of their control. Apparently the testes of every treated bird were increased in size by from 50 to several hundred per cent as the result of administration of this pituitary extract. These results can be satisfactorily given in tabular form (table 2).

The ovaries of the 5 females treated with the same extract give but little evidence from their weights that their growth was accelerated as the result of this treatment. Two of these ovaries were heavier, none were smaller, than the weights of their control would lead us to expect. This result is shown on figure 2. Other evidence of a favorable action of this extract on the ovaries is, however, provided by the fact that the oviducts of all of these 5 birds were larger than the average of their control (table 3). Other workers have shown that the action of this hormone is never directly on the uterus, but indirectly through the ovaries. With these glycerin injections, as with the transplantations described above, the ovaries of the females were much less influenced than were the testes of males.

Injection of other extracts. It remains to record briefly the results obtained from inadequate tests of two other extracts prepared as follows: Eighteen fresh anterior pituitaries from full-grown cattle were dissected from the entire hypophysis, ground in a mortar, and extracted with 0.3 per cent glacial acetic acid. The filtrate was evaporated to dryness; the heat thus applied precipitated a considerable amount of protein which, after careful washing, was separated from the filtrate. The residue from this filtrate was taken up in glycerin for injection. This extract we shall call an "acid extract." The residue from the acid extraction, together

TABLE 2

Results of daily injections of young male doves with a glycerin extract of the fresh anterior pituitary of the ox

GROUP	NUMBER	AGE	TESTIS WEIGHTS			BODY WEIGHT	
			Average	Maximum	Minimum	Start	End
		months	*mgm.*	*mgm.*	*mgm.*	*grams*	*grams*
Control	4	2.2	6.9	8.5	4.9		151
	6	2.4	6.1	8.3	4.2		149
Treated	H66	2.2	12.3			157	132
Control	7	2.8	5.9	10.0	4.1		144
Treated	D907	2.7	37.4			147	122
	D982	2.8	12.9			145	136
	D972	2.9	175.3			155	154
Control	13	3.1	8.6	18.7	3.9		152
Treated	D996	3.0	20.8			158	151
	D980	3.0	37.6			145	142
	D988	3.1	23.0			138	136
	D920	3.1	13.2			135	134
	D973	3.2	15.0			144	145
Control	7	3.4	8.4	13.0	5.3		153
Treated	D916	3.5	168.2			148	138
	D937	3.5	80.8			161	152

with the above-mentioned protein coagulum, was next extracted with boiling alcohol. The residue from this extraction was prepared for injection by dissolving or suspending it in 2 cc. of mazola to which 10 cc. of glycerin were slowly added. This extract we shall call an "alcohol extract." The injection of both of these extracts began within 36 hours of the time the cattle supplying the pituitaries were killed.

The "acid extract" was injected daily, partly subcutaneously and partly intraperitoneally, into 4 male and 4 female immature doves. Three birds each received the equivalent of 1 ox pituitary in 7 injections. Five birds each received the extract from 3 pituitaries in 14 injections. The results,

or rather the absence of results on size of testis and ovary, are shown in figures 1 and 2.

The "alcohol extract" was given in 14 injections—subcutaneous and peritoneal—to 4 male and 2 female immature doves. An examination of the weights of the gonads obtained from these birds (figs. 1 and 2) indicates that the growth of these organs was not affected by this extract.

It is probable that too small amounts of both "acid" and "alcohol extracts" were used, and that similar extracts in greater concentration should be further tested. We were stimulated to make these few tests of an acid extract by the results of Evans (1924) who found that his (unheated) acid extract "left the ovulatory mechanism intact"—that is, it did not

TABLE 3

Effects of various anterior pituitary treatments on size of oviducts in immature (not castrated) female doves

KIND OF TREATMENT	NUMBER	AGE	WEIGHT OF OVIDUCTS						
			Treated			Controls			
			Average	Maximum	Minimum	Average	Maximum	Minimum	Number
		months	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	
Transplantations	1	2.4	12.5			17.9	28.8	10.0	4
	1	3.7	18.0			70.2	211.0	20.2	5
	11	4.0	22.1	40.0	14.0	51.4	235.0	15.3	13
Glycerin extract	2	2.4	26.5	32.0	20.9	17.6	30.3	10.0	11
	1	3.1	28.8			27.4	36.7	19.0	8
	2	3.5	81.6	111.2	52.0	45.8	211.0	15.8	9
Acid and alcohol extracts	1	2.0	23.3			13.8	15.8	12.1	3
	1	2.8	25.2			19.5	24.3	15.0	8
	3	3.1	24.0	31.6	19.6	27.4	36.7	19.0	8
	1	3.5	27.3			45.8	211.0	15.8	9

prevent the growth and maturation of ova. Also, since Evans used very heavy dosage it was thought that favorable effects might be obtained by smaller dosage. We also wished to obtain some light on the question whether heat is in fact destructive to the hormone which accelerates *sexual maturity*. Nearly all of the previous work with extracts of the anterior pituitary have dealt with the hormone for *body growth*; and the more general experience, excepting that of Robertson (1925; and earlier papers) indicates a destruction of that particular hormone by heat.

Effects on the oviduct. The birds used in this study were of course not castrated, and the pituitary hormone had opportunity indirectly to affect the size of the oviducts as a result of its direct effects on ovarian growth.

Among the normal birds on which this study is based it is, however, an observed fact that until shortly before the attainment of sexual maturity the weight of the oviduct increases very little while the ovary increases very much. We may cite an example from the control data for this study which indicates the relative amount of growth in these two structures from the age of 2.0 to 4.0 months. The smallest of 3 ovaries examined at the 2.0-month stage weighed 10.3 mgm., and its associated oviduct 12.1 mgm. The smallest of 13 ovaries at the 4.0-month stage weighed 30 mgm., and its oviduct only 15.3 mgm. While the ovary increased its weight by nearly 300 per cent the oviduct increased only 26 per cent. These and other related facts that could be cited led us to expect slight effects on oviducal weight even in those few cases where the growth of the ovary was notably accelerated. This expectation is confirmed by the data of table 3. Smith and Engle (1927) also got much less marked growth of the uterus than of ovary in mice and rats, and in some cases the uterus showed no increase whatever.

The oviducts from birds receiving transplants give no evidence of enlargement; they were probably wholly unaffected by the treatment. The average of their control seems much heavier, but this results from the circumstance that a single individual in each of the advanced age groups—3.7 and 4.0 months—was closely approaching maturity and they had oviducts of most extraordinary size. The fewer treated birds, however, include no case of a bird really close to sexual maturity—the normal time of which varies between 4.0 and 12 months (though very few mature earlier than 5.5 months). The oviducts of birds treated with acid and alcohol extracts likewise fail to be differentiated notably or consistently from their controls. But the 5 birds receiving glycerin extract present a different picture. This extract was far more effective than any other treatment used in producing growth in the testes. It gave some indications of increasing ovarian size, and it here gives additional evidence of effectiveness in increasing oviducal weights. Each of these 5 glands was larger than the average of fair numbers of control.

Effects on other organs. The weight of the *thyroid* is extremely variable in the material used by us. Neither transplantations, nor acid and alcohol extracts gave any evidence of an effect on thyroid weight. The glycerin extract does appear to have caused some notable enlargements of this gland in both males and females, though this is not entirely certain. The weight of the *liver* was either unaffected or somewhat reduced in birds receiving transplants. Among the 13 males thus treated only one bird, and among 13 females only 3 birds, had a liver as large as the average of its control. Of the 16 birds injected with glycerin extract 14 showed notable enlargement of the liver—often to more than double the normal size. The acid and alcohol extracts gave no evidence of an effect on its size. The

spleen reacts quite like the liver to these treatments. The weight of the *bursa Fabricii* seems unaffected by the transplants. It was usually reduced to 30 to 50 per cent of its normal size by glycerin extract; but in 3 of 16 cases it probably suffered no reduction. It was usually, though not always, markedly reduced by acid and alcohol extracts. This organ, however, undergoes a temporary involution under many kinds of treatment, and apparently under all conditions adverse to growth and health.

Effects on body weight. Injections were usually limited to so short a period—7 to 17 days—that we have little reason to expect any marked accelerating effects on body growth. The notable point is, however, that most of the substances injected resulted in a diminished body weight; though this is doubtless largely offset by the circumstance that blank injections of glycerin alone was found to result in a slight decrease in body weight. Normally a slight amount of growth would occur during the period covered by these injections. Only one of 11 males receiving the glycerin extract—which was very effective in accelerating growth in the testis—weighed as much at the end of the test as at the beginning (table 2). On the average the body weight of these males was reduced from 147 to 140 grams. Curiously, the 4 females—whose gonads were but little affected by this extract—maintained or increased their body weight from an average of 145 to 146. The acid and alcohol extracts reduced the weight of 6 females from 136 to 130 grams; of 8 males from 139 to 131 grams.

Transplantations were usually continued over a considerable period, and in this case apparently some opportunity was offered for the growth-promoting hormone to have an effect on body growth. Data on this point are given in table 1. There is no evidence of such an effect. Values obtained at the beginning of transplantation (in birds aged 54 or more days) are practically equal to those obtained at the end of treatment. Again, in general, the weights obtained at the end of treatment do not tend to exceed those of groups of birds killed for control. The females (nos. 1–12) slightly exceed (4 grams) their special control, while the males (nos. 1–12) fall (5 grams) below their control.

Controls, and blank injections of glycerin only. The very considerable normal variation in size of testis and ovary in different immature birds of the same age makes the matter of adequate "controls" a very important one. We have sought to meet this need, first by supplying three or more controls for each treated animal; and second, by providing a number of age-groups, each of which is of some further service as controls for preceding and succeeding age-groups. Only about half of our total of control birds were killed and examined at the same time as the treated birds. During the immediately preceding year it had been found necessary to obtain precisely similar control for similar ring doves then being used in a study on the effects of the ovarian hormone on gonad size (Riddle

and Tange, 1928). In order better to show the normal size variation of the glands in these ring doves the controls taken for the two separate studies have now been combined for use in both of these studies.

We early noted an adverse reaction of the skin and tissues of the bird to injection of substances dissolved in oil or glycerin. Some of our control birds were therefore injected with glycerin only. Twelve immature birds—4 males and 8 females—were given intraperitoneally 0.3 cc. of glycerin daily during 10 days. The results were as follows: The average body weight of males decreased from 144 to 139 grams; that of females from 132 to 126 grams (for effects on organ weights see Riddle and Tange, 1928). It is evident therefore that at least a part of the adverse effect of our injections of hormone preparations on body weight may be ascribed to the use of glycerin alone. It is also clear that the size changes here found in testis and ovary are wholly unrelated to the glycerin used. It is to be noted that the age of a bird is here calculated from the date of beginning embryonic development—not from the date of hatching.

In this connection it is to be noted that from 8 of the birds receiving transplants the bursa Fabricii was extirpated before the beginning of treatment. As noted elsewhere, bursa and thymus are thought to retard the growth of the gonads in these doves, and by these operations it was sought to learn whether the absence of the bursa alone would allow the transplants to be more effective in accelerating their growth. This was found not to be the case. The gonads of the bursectomized birds—6 females and 2 males—are designated in figures 1 and 2 by periods (.) placed above the vertical bars which graphically indicate their size.

Discussion. In addition to the work cited in introductory paragraphs on rats and mice the pituitary hormone for sexual maturity has been since reported in a few other forms. Brouha and Simonnet (1927) seem to have confirmed the work on rats by daily administration of an acetone-soluble extract. They obtained premature oestrus, though it does not seem clear that they got precocious ovulation. Reichert (1928) showed that daily heterotransplants from rabbits quickly effected all the external signs of sexual maturity in a hypophysectomized dog. Earlier, Zondek and Aschheim (1927) had shown that human and bovine pituitaries were effective on mice. And Smith and Engle (1927) found that this tissue from mice, rats, cats, rabbits, and from (most) guinea pigs was effective in mice and rats. In 5 tests with immature pigeon pituitaries, given for 5 days, these workers failed to get any increased size of ovary, but got 1 case of enlarged uterus. They note also that in 2 tests with guinea-pig pituitaries, given 10 and 12 days, there was no increase in the weight of the genital system of male rats. It seems that these occasional failures —like some of our own reported here—can not be explained at present. Some of the preparations used by us were either inert or they were given

in inadequate amounts. Again, because of the prolonged period of slow growth of the bird's ovum, possibly 30 or 40 transplants are required to effect a change equivalent to that produced by 5 transplants in a mammal. It is clear, however, that these negative results are of little weight compared with the positive results. The successful tests of the present study show that the anterior pituitary bears the same relation to sexual maturity in birds as was earlier found in mammals.

With fair uniformity Smith and Engle found that transplants are less effective on immature male mice and rats than on females. In doves we have observed precisely the reverse order of effectiveness. The difference is not readily understood. We here note only that in each case it is the gonad of the homozygous sex that responds most actively; the heterozygous sex—the one that produces two chromosomal types of germ cells—responds least. Some similar instances of this sort of thing are known.

For reasons stated at the outset we are here concerned only with injections and transplantations of fresh anterior pituitary tissue; and with these only in their relation to precocious sexual maturity. Evans and Simpson (1926), by intraperitoneal injections of a NaOH extract of bovine anterior lobes which accelerated body growth in male rats, got some decrease in testis size in 2 tests and a decreased sex response to females in 7 of 10 tests. Walker (1925) made intraperitoneal injections of fresh substance into mature reproducing fowls. An inhibition of ovulation was reported. But this result can not be specific for pituitary substance. It doubtless interferes with one or another of the many endocrine changes and balances which Riddle (1927) has found associated with ovulation in the bird. In laying doves Riddle (1923) was able to inhibit 90 per cent of the normal number of ovulations by injections of moderate doses of insulin.

While our knowledge of extraction methods for the various hormones of the anterior pituitary remains so unsatisfactorily known it would be of great advantage if all extracts made were carefully tested for their action both on growth and on sexual maturity. Teel's (1926) extract of bovine pituitaries, made by Evans' method, seemed to lack the gonad-stimulating hormone. But Zondek and Aschheim showed that the fresh bovine glands contain this hormone, and the glycerin extracts used by us have proved highly potent, at least on males. We find glycerin extracts of bovine anterior lobes much more effective in causing gonadal growth in doves than are 9 to 25 daily transplants from other doves. By means of this extract, or of others in which the protein content is reduced, we hope later to be able to rear treated doves and observe the exact age at which a precocious sexual maturity is attained. In these further studies we hope also to make more adequate tests of effects on growth than has been possible with the short periods of treatment used in the present investigation.

Attention should here be called to the very different response of the

testes and ovaries of doves to the injection of this hormone, as compared with the use of the hormone of the ovary and placenta (Riddle and Tange, 1928). In the latter case both testes and ovaries were notably reduced in size. Here, transplant moderately increases, and glycerin extract greatly accelerates, testis growth; while both of these either favor ovarian growth or leave it unrestricted. Again, our injection of acid or alcohol extracts of anterior pituitary are merely without effect on the growth of testis or ovary. The ovarian hormone, and this particular pituitary hormone, produces opposite effects on the growth of the gonads of doves. The growth of testis and ovary in these birds is favored by a product of the anterior pituitary. This contrast is drawn here particularly because precisely the same control animals have been used in making tests on these two hormones.

In a consideration of the forces which may *normally* oppose the pituitary hormone in hastening the appearance of sexual maturity in an animal the above-mentioned ovarian hormone does not enter, since it seems not to be formed until maturity is accomplished. But other data recently obtained by Riddle (1928) supply some evidence that the thymus and bursa Fabricii exert an action opposite to that of the pituitary on the growth of testis and ovary in these same animals. In the case of ring doves therefore we now have some evidence concerning one agent which presses for early sexual maturity, and another agent which tends to push the time of sexual maturity to a later period. Here there is reason for regarding the actual time of sexual maturity as a resultant of these two forces.

SUMMARY

Anterior pituitaries of mature doves and cattle were studied as sources of a hormone capable of inducing gonad growth and precocious sexual maturity in immature doves. Tests were made on the effects of transplantation of 509 dove pituitaries, and of the injections of glycerin, acid and alcohol extracts of bovine glands.

Daily homeotransplants produced increased growth in the testes of immature male doves. A less marked effect was found in some only of the ovaries.

The intraperitoneal injection during 7 to 17 days of a glycerin (commercial) extract of fresh unfrozen bovine anterior lobes increased the testis size of 11 immature males by 50 to several hundred per cent. Similar dosage in 5 females probably produced a slight increase in the size of some of their ovaries.

The acid and alcohol extracts of fresh bovine glands were without effect; but the dosage may have been inadequate.

Body weight was usually unaffected or adversely affected during the period of transplantation or injection. Birds receiving glycerin extracts

usually had enlarged thyroids, livers and spleens. Other treatments were probably without effect on organ weights.

The work of others dealing with the anterior pituitary as the source of the hormone which accelerates the attainment of sexual maturity is confirmed, and is extended to birds.

Earlier work of this laboratory has provided some evidence that in these ring doves the bursa Fabricii and the thymus retard gonad growth and sexual maturity. In this same animal the anterior pituitary is now identified as the source of a hormone which acts in the opposite direction. Here there is reason for regarding the actual time of sexual maturity as a resultant of these two forces.

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THE RÔLE OF TOXINS IN PARATHYROID TETANY

II. Tissue Changes in Parathyroid Tetany and in Guanidine Poisoning

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Since Koch (1913) isolated methyl guanidine from urines of thyro-parathyroidectomized dogs, much work has been done to establish a connection between this toxic base and the tetanic syndrome. This investigator (Koch, 1913) also reported the tissue changes which he observed in dogs dying of parathyroid tetany. Burns, Paton, Findlay et al. (1917), especially, have published the results of various experiments in which they endeavored to prove the close association of this toxic base to parathyroid tetany. They concluded that the parathyroid secretion in some way regulates guanidine metabolism in the body, an excess of which results from parathyroid deficiency and hence, tetany. In their voluminous report they conclude that the symptoms of tetania parathyreopriva and the symptoms produced by the administration of guanidine and methyl guanidine are identical. Dragstedt et al. (1923a, 1923b, 1926) in a series of investigations also attempted to demonstrate the toxin theory of parathyroid tetany.

Susman (1926) reported microscopical changes in the parathyroid glands as a result of intravenous and subcutaneous injections of guanidine nitrate. No microscopic study was made of the other organs of the body. This work of Susman prompted us to make microscopic studies of the various glands of animals dying of parathyroid tetany and of guanidine poisoning. We did this with the object of making a comparative study of the microscopical changes resulting from parathyroid tetany and from guanidine poisoning. We felt that such a comparison would be relevant and would yield some valuable information regarding the visceral changes in these two syndromes.

Methods. Two rabbits, two cats and two dogs were injected subcutaneously with guanidine hydrochloride 0.4 gram, 0.2 or 0.3 gram per kilogram of body weight, respectively. These animals were allowed to die and were then immediately autopsied. Pieces of the various viscera were fixed in 10 per cent formaldehyde. Two dogs were also fed 4 grams of guanidine per kilo of body weight by stomach tube.

Three dogs were thyro-parathyroidectomized under aseptic technique using ether anesthesia. These dogs were allowed to die from tetany. They were likewise autopsied immediately following death and portions of the viscera preserved as above.

The tissues obtained in this manner we dehydrated in alcohol and cedar oil and subsequently infiltrated with and imbedded in paraffin. They were cut in sections of 5 to 10μ and stained by the ordinary eosin-hematoxylon method.

Typical protocols and histological findings:

Dog C. Female, black, medium age, weight 8 kilograms.

December 13, 1927. 10:15 a.m. Injected 3 grams of guanidine hydrochloride subcutaneously. Within the next hour the dog had hypernea, salivated and vomited but did not have the marked muscular spasms so evident in tetany.

3:15 p.m. Dead.

3:30 p.m. Autopsied. No marked gross pathology. There were a few small stones in the pelvis of each kidney.

Microscopical examination: Kidney—Cells of tubules often appear to have been drawn away from the basal membrane. Cells are swollen and in some cases have ruptured the cell membrane. Simple necrosis with nuclear changes. Glomeruli appear normal. No congestion.

Liver—Great fatty degeneration throughout entire lobule. At isolated points there is hemorrhagic congestion. The nuclei appear fairly intact even of the fatty cells.

Spleen—Some congestion otherwise no perceivable changes.

Adrenal—Some simple necrosis of all areas. No congestion.

Dog T3. Large brown and white male, weight 12 kilograms.

November 30, 1927. Thyro-parathyroidectomized.

December 3, 1927. 8:00 a.m. Dead. Autopsied immediately. No apparent gross changes. Microscopic examination: Kidney—Great amount of active congestion of cortex including glomeruli. Cells of convoluted and straight tubules show simple necrosis with bursting of cellular membrane and withdrawal of cytoplasmic debris from basal membrane to center of lumina of tubules. Karyorrhexis and karyolysis of nuclei of cells of tubules. Glomeruli were normal except for congestion.

Liver—Decided congestion, simple necrosis of liver cells. Orderly arrangement of hepatic cords no longer present.

Spleen—Congestion. Active hyperemia.

Adrenal—Decided active congestion. Cells of area glomerulosa fragmented, also evidence of simple necrosis. Same for area fasciculata. Great active congestion of medulla with simple necrosis.

Testis—Tubules and epididymis normal.

Dog S1. Young male, weight 7 kilos.

11:00 a.m. Gave 35 grams of guanidine hydrochloride by stomach tube. Vomited some of the material. The symptoms, such as salivation and muscle tremors, were similar to those seen after subcutaneous administration of the same compound.

2:00 p.m. Dead. Autopsied immediately. Microscopic examination: Kidney—The convoluted tubules showed simple necrosis with pyknosis, karyolysis, karyor-

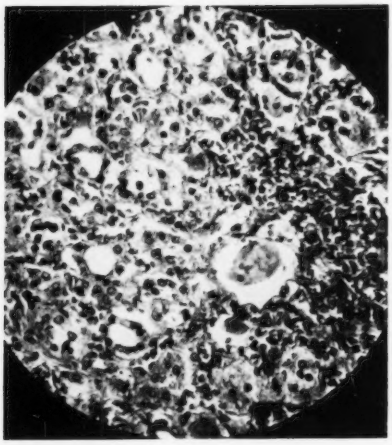

Fig. 1

Fig. 2

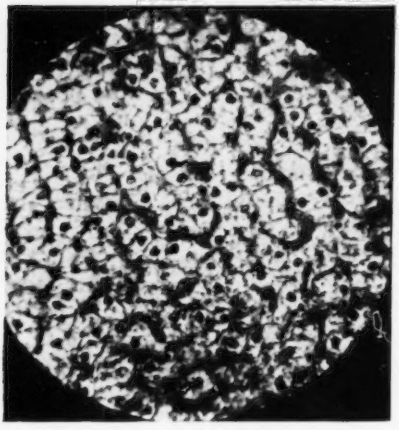

Fig. 3

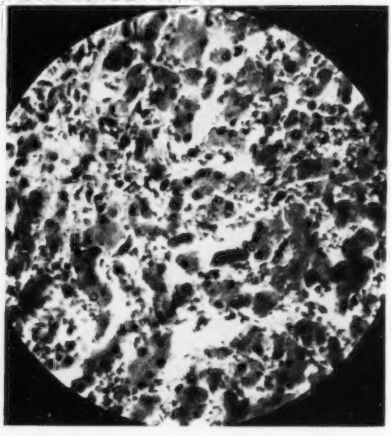

Fig. 4

Fig. 1. Kidney. Dog T3. Parathyroid tetany. Necrosis and cytolysis is very evident. The cellular casts are undergoing hyalinization. Lymphocytic infiltration is also present.

Fig. 2. Kidney. Dog B. Guanidine poisoning. The tubules are necrosed but the glomerulus is normal.

Fig. 3. Liver. Dog C. Guanidine poisoning. The liver has undergone fatty degeneration.

Fig. 4. Liver. Dog T4. Parathyroid tetany. Necrosis and albuminous degeneration.

rhexis. The cytoplasm was swollen so as to break the cellular membrane and collect in the lumina of the tubules. The straight collecting tubules exhibit some nuclear changes but no cytoplasmic changes. The glomeruli were normal. The liver has extravasated blood throughout. There is some necrosis at the peripheral portion of the lobule. Albuminous degeneration is present. At isolated points there is fatty degeneration. The adrenals are normal. The parathyroids have a normal arrangement of cells. There were no fatty globules present nor was there any evidence of hyperplasia.

The microscopic findings in the parathyroids of the dogs dying from guanidine poisoning were as follows:

The structure was of the anastomosing column type. All three types of cells were found with the dark chief cells predominating. There were no fat globules nor colloid present. No degenerative processes were noted but there was slight congestion. In the rabbits to which guanidine had been administered, the parathyroids were of the compact type. The dark chief cells predominating with no increase in the oxyphilic cells. Numerous fatty globules were noted throughout the entire gland. There was a great amount of colloid present.

Discussion. The tissue changes reported by Koch (1913) were as follows: Kidneys showed congestion and hemorrhage especially of the cortex. In some cases the kidneys were anemic and the medulla was congested. The glomeruli were congested and hemorrhagic, forming blood islands. Bowman's capsules in some cases were destroyed and the cells of the convoluted tubules were degenerated. The spleens were congested and contained blood pigment, some of the cells showing chromatolysis. The vessels of livers, he noted, contained fragmented erythrocytes and normoblasts and also erythrocytes staining with eosin. Liver sections showed large mononuclear cells containing eosinophilic globules. The hepatic cells showed fatty degeneration. The lungs were edematous and congested with large mononuclear leucocytes present. The intestinal tract was congested and presented degenerated epithelium at the pyloric end of stomach and in the duodenum.

That parathyroid tetany causes congestion of all the organs examined, except the testes, was the most consistent and outstanding change which we observed. The kidneys of dogs T_2, T_3 and T_4, dying of parathyroid tetany were actively congested, especially the cortex. The medullae were also involved. The glomeruli were greatly congested but in no case did we find any degenerative changes in them. Koch (1915–16) reported that some of the glomeruli were hemorrhagic and that others had lost Bowman's capsule. The epithelium of the convoluted tubules as well as the epithelium of the straight collecting tubules were necrosed showing karyolysis and karyorrhexis. In T_3 and T_4, the cells of the tubules were swollen to such an extent as to burst the cellular membrane and extravasate the cytoplasmic content into the lumina. The livers of these dogs were also actively con-

gested. The hepatic cells in case of dog T_3 were necrosed with karyolysis and karyorrhexis. In dog T_2 there was some slight fatty degeneration which was not localized. The other two livers showed no fatty degeneration which is not in accord with Koch's (1913) findings. During the generalized extreme muscular contraction accompanying a tetanic attack, blood is evidently forced from the muscles to accumulate in the splanchnic viscera. From this the active congestion probably results. The degenerative changes noted are probably due to this congestion inasmuch as the degree of the degenerative changes is in direct proportion to the time the dog lived following thyro-parathyroidectomy. In dog T_2 that lived nearly two weeks in a tetanoid condition before being sacrificed, the degenerative changes of the liver especially were intense, showing some fatty degeneration. In dogs T_3 and T_4 that lived three and six days respectively, the necrotic changes are less pronounced. If degeneration resulting from congestion is relative to the duration of the congestion, our results bear this out precisely.

Guanidine administration, either subcutaneously or by means of a stomach tube to cats, dogs or rabbits in fatal doses, produces entirely different histological changes than parathyroid tetany causes. The kidneys showed degenerative changes of the epithelium of the convoluted and straight collecting tubules. The cells were swollen and in some cases had ruptured to extravasate the cytoplasm into the lumina of the tubules. The glomeruli were not affected. The livers, in every case, showed fatty degeneration of the hepatic cells at the periphery of the lobule, that is, at the portal areas. The fatty degeneration became less pronounced as it proceeded from the portal system to the central vein, the cells around the central vein being swollen and showing evidences of simple necrosis with nuclear changes. In some cases, as cat A, rabbit A and dog B, there were evidences of some leucocytic infiltration. Blatherwick et al. (1927) reported microscopical findings in the kidney and liver resulting from injections of synthalin, a derivative of guanidine. They reported that the cells of the convoluted tubules of the kidneys were damaged to the extent of complete destruction of the cells to form granular debris in the lumina and that there were no noteworthy changes in the glomeruli. The livers showed moderate fatty changes. Simola (1927) also reported fatty degeneration of the liver and nephrotoxic changes in the kidney with large doses of synthalin. Szczeklik's (1927) findings are somewhat similar to those of Simola. When synthalin was given on three successive days, Bodo and Marks (1928) found extensive and severe fatty dengeration at the periphery of the liver lobule. The spleens in our dogs were apparently normal although in some cases there was slight congestion. The adrenals showed simple necrosis of the cells of both cortex and medulla. The testes and epididymides were normal.

Susman (1926) reported results on parathyroids of normal rabbits in which he injected guanidine nitrate declaring that these injections caused a

depletion of fat globules in the parathyroids within 18 hours, to be restored in 48 hours. Also, on repeated injections in which there was maintained a degree of hypertonicity of the muscles, there resulted hypertrophy and hyperplasia of the parathyroid cells with an increase of the acidophilic cells and a depletion of the large fat globules. He concluded that these results gave evidences of an increased activity of the parathyroid gland because of the injections of guanidine nitrate. This may not be a correct conclusion because Marine (1928) believes that the mode of secretion of the parathyroid glands has not been established. Susman's conclusions are not complete because he failed to examine the other tissues of the body which would be necessary in order to ascertain whether or not such changes as he reported were in truth increased functional changes or the result of the toxic action of the base. We examined sections of the parathyroid glands from dogs dying of guanidine poisoning and found no appreciable changes. There were no fat globules discernible. However, in the parathyroids taken from a rabbit dying of guanidine poisoning we did note fat globules. Hence, it would seem that the matter of fat globule content of the parathyroid gland is a variable factor depending on the species concerned.

Our observations lead us to conclude that microscopical changes are produced in the other tissues of the body in guanidine poisoning. In other words, the changes Susman observed in the parathyroid glands cannot be purported to indicate functional increase in view of the fact that marked histological changes are present in other tissues. It is natural to assume that these changes are the result of the action of the toxin, guanidine, on the tissues directly, parathyroids included.

The tubules of the kidneys of the tetany animals were degenerated and the glomeruli congested. In kidneys of those animals dying of guanidine, there was no congestion but only a degeneration of tubules with some leucocytic infiltration, but no effect on the glomeruli. The livers of the tetany dogs were congested presenting in two cases simple necrotic changes of the hepatic cells. Whereas, the livers of the animals dying from a fatal dose of guanidine hydrochloride showed fatty degeneration at the periphery of the lobule around the portal areas indicating that the cause of the change was a toxin contained in the blood producing its greatest damage at the point that it enters. The livers in these cases were not congested. As for the spleens and adrenals, they were greatly congested in the tissues of parathyroid tetany dogs, the adrenals presenting varying degrees of necrosis but those of the animals dying of guanidine poisoning were not congested and only simple necrosis of the adrenals was perceived.

The symptoms of parathyroid tetany and of guanidine poisoning seem to us to be quite different. We know that there are some muscle tremors in both conditions as well as salivation and hyperpnea. In tetany the sequence of events is different from that of guanidine poisoning. The muscle

tremors, salivation and slight hyperpnea in tetany is followed by decided hyperpnea and a marked spasticity of the entire skeletal musculature whereas in guanidine poisoning the animal becomes more and more depressed, finally dying in a state of profound depression. In cats, Paton and Findlay (1917) state that the symptoms are identical. But Swingle and Nicholas (1924) found the symptoms produced in cats by either oral administration or subcutaneous injections of guanidine compounds, were not comparable to tetany of parathyroid origin.

SUMMARY

Parathyroid tetany produces a marked congestion of the viscera. This congestion is not found in guanidine poisoning. Necrosis was present in both conditions. Fatty degeneration of the liver was marked in guanidine poisoning but absent in tetany. Our observations do not support Susman's conclusions. The symptomatologies of the two syndromes are quite dissimilar.

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THE EFFECT OF FATIGUE ON HEAT RIGOR AND CHLOROFORM RIGOR IN THE GASTROCNEMIUS AND SARTORIUS MUSCLES OF THE FROG

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INTRODUCTION AND METHOD. Will a muscle in complete fatigue show the same death shortening as non-fatigued muscle under the conditions that produce heat rigor or chloroform rigor in the normal non-fatigued muscle? The writer first became interested in this question in 1923. The only paper found then, dealing directly with the question, was one by Latimer (1898). She found that the fatigued gastrocnemius muscle of the frog entered into rigor at a lower temperature and shortened much less than its mate which had not been fatigued.

The question seemed of such importance in connection with the entire problem of the physiology of skeletal muscle that it was decided to study it with further experiments. Latimer had fatigued through the nerve only. It is well known that when fatigue has resulted through nerve stimulation (carried out ever so carefully) the muscle will usually still respond if stimulated directly. Suppose that the muscle should be excised first, and then stimulated to *complete* fatigue through direct stimulation. Would the same modification of rigor result as when, in a muscle with its circulation intact, fatigue had been produced through nerve stimulation alone? Also, for obvious reasons, it seemed desirable in this connection to fatigue a muscle in an atmosphere of nitrogen or carbon dioxide devoid of oxygen and compare it in rigor with its normal mate. Accordingly apparatus was devised, designed to hold a frog's two excised gastrocnemius muscles (or the two sartorius muscles) attached to different levers of equal length. The arms of the two levers were made exactly the same and they could be readily adjusted to write, the one a short distance behind the other, upon the same base line. The muscles could be enclosed by the same moist chamber, in a large water jacket, when heat was to be applied; or, in the other type of experiment, moist CO_2, for example, could first be passed through the chamber around the muscles, until the time arrived for causing rigor. On the tendon at each end of each muscle was securely fastened a small loop of waxed silk. The muscles were attached to hooks, in the apparatus, by means of these loops on the tendons,

and so it was possible to remove a muscle and weigh it, at any time during an experiment. The loop at one end of a muscle was placed over a stationary hook, which could be adjusted by a set-screw outside of the moist chamber. The loop at the free end of the muscle was attached to a platinum hook, which connected by fine magnet wire (no. 36 on the Brown and Sharp standard gauge) to the lever. It was found that the small waxed silk loops used did not suffer measurable changes in length under the treatment to which they were subjected in the experiments. No difficulty was experienced except that above about 58°C. the tendons of the muscle sometimes became so tender as to be cut by the silk—even though the lever pull on the muscle was not over two grams. The really significant temperatures, however, for heat rigor are very much lower. Arrangement could be made to stimulate one excised muscle, either through its nerve or directly, as desired. Great care was used to keep the two muscles under the same conditions of temperature, moisture, etc.

In some respects Latimer's plan of fatiguing the muscle in the animal with the circulation intact seemed most desirable. However, this plan involved the excising of the two muscles later, before they were attached to the "rigor levers" and brought to the condition of rigor. Experience soon showed that the process of fatiguing the muscle often caused it to assume a different length, in the fatigued state, under the same conditions of tension and temperature, than it had before the fatiguing process began. If it had been fatigued very slowly and under considerable tension, it might show great relaxation. On the other hand, it was found that the fatiguing process more often caused the muscle to hold at a little shorter length, under the same tension, than before fatigue. Even when other conditions were kept as nearly as possible the same, the result seemed to vary somewhat with different frogs. Therefore, in order to make sure that the muscles were really placed upon a comparable base line, it seemed advisable carefully to excise both muscles first, attach them to their levers, and adjust them to the same base line under exactly the same conditions, and then to fatigue one of them in moist air. Only occasionally, as seemed advisable, during the time one muscle was being fatigued, were the muscles both wet with Ringer's solution. The Ringer's solution used in all the experiments, and referred to hereafter as Ringer's solution or as ordinary Ringer's solution was made as follows: NaCl 6.5 grams, KCl 0.14 gram, $CaCl_2$ 0.12 gram, and $NaHCO_3$ 0.20 gram, dissolved in 1000 cc. of distilled water. Its pH was always about 8.4. Sometimes after the muscles were adjusted to the base line, an extra weight (varying with the size of the muscle) was placed on the lever arranged for isotonic contractions, during the time the muscle was being fatigued. This extra weight was removed at the end of the fatiguing process, so that both muscles were under the same tension again before conditions were modified to bring about rigor.

Effect of fatigue on heat rigor. First, a series of experiments was carried out to test the effect of previous fatigue upon heat rigor. These experiments confirmed the general finding of Latimer that a fatigued frog muscle will pass into heat rigor at a lower temperature than its mate which has not been fatigued and that the former will not shorten so much as the latter.

Since Latimer has already tabulated her results in the paper cited, it will be sufficient here to briefly summarize results of my experiments and then point out in what respects they differ from hers.

In ten experiments using excised gastrocnemius muscles of the frog, it was found that the fatigued muscles began the heat rigor shortening, at an average temperature, 8.7°C. lower than their non-fatigued mates; and that they shortened, on the average, 24.3 per cent less than their mates. In case of excised sartorius muscles, the difference in temperature at which rigor began in the fatigued and non-fatigued muscles was not nearly so great (1.5° to 0.5°C. difference, only), but even when the temperature at which the fatigued sartorius started to shorten was almost as high as that at which its non-fatigued mate began to shorten in heat rigor, the fatigued muscle shortened the more rapidly *at the beginning*, and was only later overtaken and surpassed by its non-fatigued mate. The fatigued sartorius muscles, on the average, shortened 40 per cent less than their normal mates—the difference being greater, in this respect, than was found for the gastrocnemius muscles. Latimer did not use the sartorius. From her table 1, however, calculation shows that the fatigued gastrocnemius muscles began shortening at an average temperature 9.9°C. lower, and shortened 53 per cent less, on the average, than their normal mates—a little more than twice as large a difference as the average difference in shortening (24.3 per cent) reported above for the ten experiments using excised gastrocnemius muscles.

Latimer's method required one to four and one-half hours for the fatiguing process. In the experiments reported here for excised gastrocnemius muscles, fatigue was brought about as quickly as possible without throwing the muscle into extensive contracture. The time varied, usually, between thirty and fifty minutes—in extreme cases the muscles might be completely irresponsive, even to direct tetanizing stimuli, in five minutes. It was found that if the excised muscle was fatigued carefully over a longer period—or after *complete* fatigue, if the muscle was allowed to stand in moist air for a time (2 hours in one case)—before heat was applied for rigor, larger differences in shortening could be obtained. Even with these precautions 38 per cent was the largest difference obtained in heat rigor using excised gastrocnemius muscles. As pointed out above, a fatigued muscle is often just a little shorter than, under the same stress, before fatigue. The method used by Latimer, therefore, which led to placing the base line of the fatigued muscle upon that of the non-fatigued muscle for comparison,

would tend to give larger average differences in the rigor shortening of the two gastrocnemius muscles than those reported here; this perhaps may account in part for the smaller average difference in shortening, between non-fatigued and fatigued muscles, obtained by my method. However, the variation in difference of shortening between a fatigued gastrocnemius muscle and its normal mate, as calculated from results in Latimer's table 1, is great—the smallest difference is found to be 30 per cent and the greatest difference 72 per cent. These extreme variations seem to indicate the possibility that where the difference is very great the fatigued muscle may have been in a stage of partial rigor already when placed upon the base line. This possibility is avoided where both muscles are excised and their attached levers are placed upon the same base line before the fatigue of one muscle is begun.

Some experiments were tried in which one gastrocnemius muscle was fatigued in moist air, at a room temperature of about 18°C., slowly, over a period of 50 minutes or so; then the stimulus was continued several minutes after the muscle ceased to respond, and after heat began to be applied, right up to the moment when rigor began; but this did not seem to modify the final result as already stated. As Latimer has pointed out, if the two muscles are allowed to stand in moist air at a room temperature of 18° to 19°C., spontaneous rigor (rigor mortis) begins several hours earlier in the fatigued than in the non-fatigued gastrocnemius muscle. But this further fact must be added: If the room temperature of the experiment stood as high as 23° to 24°C., even, it was observed that a slow shortening (i.e., a slow rigor) of the fatigued muscle might begin almost immediately upon the completion of the fatiguing process—particularly if the stimuli had been very strong.

It should be made clear, now, that the heat rigor shortening considered so far is completed at a comparatively low temperature—on the average at between 43° and 45°C.—and it begins, as a rule, between 35° and 38°C. in the normal muscle. However, if heat is continued, in case of the gastrocnemius muscle, a second step in shortening begins in the neighborhood of 48°C. to 52°C.; it is usually complete at 54° to 58°C. Finally, when the temperature has risen to between 58° and 74°C., a third shortening begins to lift the lever again—provided the tendon does not pull off. This third shortening is not complete until the temperature rises to between 75° and 80°C. While the temperature at which heat rigor begins in a muscle varies somewhat with different frogs, it begins at the same temperature in any non-fatigued normal muscle and its mate which have been excised under the same conditions. When the sartorius muscle was used, the second step in shortening was never conspicuous and often did not show at all, but the first and third steps in shortening appeared at the temperatures described.

The first of these shortenings (i.e., the first step in the heat rigor curve) was the only one studied by Latimer, and it seems to be the most significant of the three steps in connection with the study of the heat rigor curves of fatigued and non-fatigued muscles.

In the experiments reported here, the largest average percentage difference in heat rigor shortening was found at the end of the first step in shortening—viz., the fatigued gastrocnemius muscles shortened 24.3 per cent less, on the average, than their normal mates. At the end of the second step in shortening, the similar difference was only 9.5 per cent; and at the end of the third shortening, the average difference (of five sets of muscles whose tendons held) was a little under 5 per cent. That is, the shortening of the fatigued muscle during the second and third steps was comparatively a little greater than the shortening of the non-fatigued muscle during the corresponding steps. Brodie and Richardson (1899) pointed out the three steps in the heat rigor shortening of normal muscle. Also they observed that fatigued muscle passed through the same three steps during heat rigor. Apparently, however, their method did not enable them to make a close comparison between the heat rigor curve of a normal muscle and its previously fatigued mate, because on page 145 (l.c.) they state: "We found that a completely fatigued muscle, when heated, gave contractions quite corresponding to those yielded by a fresh muscle."

The first step in shortening was clearly affected *directly*—(i.e., reduced in extent and caused to start at a lower temperature) by fatigue. Was fatigue of the muscle directly responsible for the relative increase in shortening of the second and third steps of heat rigor? In case of the third step in shortening it was found that it, almost certainly, cannot be considered as a direct effect. Trial experiments with pieces of gastrocnemius tendon (i.e., tendon of pure connective tissue—no muscle included) showed that no change in length occurred with rise in temperature until a temperature between 58° and 65°C. was reached, when a pronounced shortening began, corresponding with the third step described in heat rigor shortening of muscle. Brodie and Richardson (l.c.) have credited Engleman and Herman with first calling attention to the shrinkage of tendon, under the influence of heat, in 1873. It can not be doubted that the third step in heat rigor shortening of the muscle bundle is really due to a shrinkage of the muscle's connective tissue, which is caused, in some way, by the heat. And it does not seem likely that fatigue of the muscle could influence its connective tissue directly to cause the effect noted. A more reasonable explanation seems to be that both the second and the third steps in shortening are actually unaffected directly by fatigue of the muscle, but develop an equal stress in the fatigued muscle and in its normal mate. Then, the partially contracted bundle of fatigued muscle, being less firm (because it has not contracted so much as its non-fatigued mate at the end of the first

period of shortening) can now be compressed and shortened a little more than its firmer mate, by the equal tension developed in the muscles during the second and third steps of heat rigor. No evidence is gained here as to what causes the second step in heat rigor; but if there is a real causal similarity between the shortening of muscle in normal contractions following stimulus, and heat rigor shortening (except that the latter is irreversible) then that similarity lies particularly with the first step in heat rigor shortening—i.e., the step which is directly affected by previous fatigue. Brodie and Richardson (l.c.) and Halliburton (1904) sought to connect the steps in heat rigor with the coagulation of different proteins in the muscle protoplasm. However, Meigs (1909) and others have published evidence to show that although there may be some measure of coincidence between the temperature of coagulation of the proteins in skeletal muscle and the temperature at which the steps in shortening occur in that muscle, the relationship can not be causal in every case.

Heat rigor shortening as influenced by after-treatment of fatigued muscle with oxygen. Following the work of Fletcher and Hopkins (1907) on the lactic acid produced in uninjured excised amphibian muscle in fatigue and in heat rigor at 40°C., A. V. Hill (1912) studied the heat production in surviving frog muscle with a differential micro-calorimeter and connected the heat production during the processes leading to chloroform or to heat rigor with the chemical processes which liberate lactic acid under the same conditions. He showed, further, that if fatigued muscle was exposed to pure oxygen for 8 to 10 hours, the lowered heat-production of rigor in such muscle would be restored to the initial high amount usual in fresh muscles, just as it was found by Hopkins (l.c.) that pure oxygen diminished the amount of lactic acid present in fatigued muscles without affecting the "lactic acid maximum" of heat rigor. Moreover, Hill (1913) made the direct suggestion that the processes of muscular contraction are due to the liberation of lactic acid (the lactic acid being rebuilt into its precursor, after the contraction, by the use of oxygen); and that the heat liberated by a muscle contracting in the complete absence of oxygen is "due to the break down of lactic acid precursor, and is the same in nature as the heat production of rigor."

In line with these findings and suggestions, it was decided to find whether a completely fatigued muscle, exposed to pure oxygen for 8 to 10 hours, would have its ability to shorten in heat rigor so restored as to equal that of its non-fatigued mate. As already stated, this investigation had shown previously that the completely fatigued muscle did not recover, in that respect, on standing in air.

In order to test the effect of pure oxygen treatment on the ability of previously fatigued muscles to shorten in heat rigor, eleven experiments were carried out. In six of these, gastrocnemius muscles were used, and

TABLE 1

Comparing fatigued frog muscles, treated with pure oxygen, with their non-fatigued mates under the same conditions, as to heat rigor shortening

EXPERIMENT NUMBER	CONDITION OF MUSCLE	TIME USED IN THE FATIGUING PROCESS	HOURS IN O_2 AFTER ONE MUSCLE WAS FATIGUED	TEMPERATURE AT WHICH RIGOR BEGAN	TEMPERATURE AT WHICH "FIRST STEP" OF RIGOR ENDED	EXTENT OF SHORTENING	PER CENT WHICH FATIGUED MUSCLE SHORTENED LESS THAN ITS MATE
Gastrocnemius muscle							
		minutes		*°C.*	*°C.*	*mm.*	
286	Fatigued	35	6½	31.0	46.0	42	
	Non-fatigued	0	6½	36.0	46.0	61	31.0+
287	Fatigued	25	8	32.0	44.5	53	
	Non-fatigued	0	8	36.5	44.5	60	11.6
288	Fatigued	33	14	30.0	45.0	59	
	Non-fatigued	0	14	36.0	45.0	66	10.6
289	Fatigued	50	15½	32.0	45.0	58	
	Non-fatigued	0	15½	35.5	45.0	69	15.9
290	Fatigued	30	11½	30.5	45.0	59	
	Non-fatigued	0	11½	36.5	45.0	64	9.3
292	Fatigued	40	12½	31.5	44.5	63	
	Non-fatigued	0	12½	36.5	45.5	67	6.0
Average for experiments nos. 287 to 292 inclusive							10.7
Sartorius muscle							
291	Fatigued	40	5	35.5	44.0	52	
	Non-fatigued	0	5	35.5	45.0	45	
293	Fatigued	33	5½	35.0	43.0	83	
	Non-fatigued	0	5½	35.5	44.5	105	20.9
294	Fatigued	20	4¾	38.0	45.0	38	
	Non-fatigued	0	4¾	38.0	46.0	54	29.6
295	Fatigued	18	4½	36.0	44.0	59	
	Non-fatigued	0	4½	36.0	44.0	51	
296	Fatigued	43	4	36.0	44.0	102	
	Non-fatigued	0	4	36.0	45.0	123	17.0
Average for experiments nos. 293, 294 and 296							22.5

in the other five experiments sartorius muscles were used. A pair of muscles was carefully excised from a frog, attached to the levers of the apparatus already described, and adjusted to the same base line—each under the same tension of about two grams. The muscles were then enclosed in a chamber of pure moist oxygen and a slow stream of oxygen was kept bubbling through the chamber from a pressure tank. One muscle was now fatigued by direct stimulation with maximal make-break shocks so spaced as to avoid contracture. Twenty grams extra were used on the lever during the fatiguing process. The other muscle was kept at rest under the two gram tension in the same chamber. The chamber was maintained at 16° to 18°C. throughout the experiment up to the time when heat rigor was started. In only one experiment of this series was a higher temperature of 25.5°C. maintained in the chamber (viz., expt. 286). When fatigue was complete (so that a short maximal tetanus did not cause contraction) the stimuli were discontinued, and about thirty minutes later the extra twenty gram weight was removed. The flow of moist oxygen was continued, however, for a definite number of hours in each experiment. Then, as the oxygen was shut off, the temperature of the chamber was raised to cause heat rigor; and records of the rigor shortening of the two muscles were obtained on a slowly moving drum. The extent of shortening of the two muscles could thus be accurately measured. Results are recorded in table 1. As will be observed, the actual time muscles were left in the oxygen, after the fatiguing process, was purposely varied in the different experiments between four hours and fifteen and one-half hours. The thicker gastrocnemius muscles were left in the oxygen longer than the thin sartorius muscles. It will be remembered that in the ten experiments where one gastrocnemius muscle of each pair was fatigued in air, followed by heat rigor in both muscles (carried out in air) the fatigued muscles shortened 24.3 per cent less, on the average, than their non-fatigued mates —38 per cent being the largest difference (p. 133). Now, in case of the one experiment in table 1 where the warm room temperature of 25.5°C. was maintained, the fatigued oxygen treated muscle shortened 31 per cent less than its normal mate in heat rigor. There was apparently no recovery due to oxygen treatment at that temperature. In case of the other five experiments, with gastrocnemius muscles, in which the lower temperature of 16°C. to 18°C. was used, the fatigued oxygen treated muscles shortened only 10.7 per cent less (average) than their non-fatigued mates. This figure indicates that they had made a little more than a 50 per cent recovery in the pure oxygen. Two of the fatigued oxygen-treated sartorius muscles (expts. 291 and 295) shortened *more* than their mates. No explanation is offered for this, but the result can be attributed to no fault in carrying out the experiment in either case. The other three fatigued oxygen-treated sartorius muscles averaged only 22.5 per cent less than their

mates in the heat rigor shortening. This must represent a decided recovery of nearly 50 per cent, due to the oxygen treatment, since the average shortening for fatigued sartorius muscles in air was 40 per cent less than their normal mates during heat rigor.

The average difference in the temperature at which rigor began in the fatigued and the non-fatigued muscles appeared to be lowered slightly, also, by the oxygen treatment. Whereas the fatigued gastrocnemius muscles which went into heat rigor in air, began to shorten at a temperature 8.7°C. lower (average) than their mates, the fatigued oxygen-treated gastrocnemius muscles began rigor, on the average, only 5°C. lower than their non-fatigued mates. The oxygen-treated sartorius muscles began rigor at practically the same temperature as their mates.

Heat rigor of muscles fatigued in pure CO_2, or in nitrogen, compared with that of their unfatigued mates under the same conditions. It was known that excised frog muscle could be fatigued to the point where it would not respond to stimulus much more quickly in an atmosphere devoid of all oxygen than in ordinary air. In three experiments carried out to test this, it was found that under the same kind of stimulation, lifting the same weight, about three times as many contractions were required to bring about fatigue of an excised muscle in air as were required to fatigue its mate in CO_2. That is, the condition of fatigue could be induced, in the absence of oxygen, with the expenditure of less of the stored fuel energy of the muscle than was the case when fatigue was induced in the presence of oxygen in air. It seemed desirable, therefore, to obtain a comparison between the extent of heat rigor shortening of muscles fatigued in the absence of oxygen (in pure CO_2, for example) and that of their non-fatigued mates, in order that it might be checked against the similar comparisons already made in air and in pure oxygen.

In conducting the experiments for this comparison, the muscles were adjusted, in the chamber of the apparatus, with their writing levers on the same base line in pure CO_2, and then a stream of moist CO_2 was made to flow through the chamber continuously until heat was applied for heat rigor. The process of fatiguing was not begun until both muscles had been in the CO_2 chamber for ten minutes. Results are given in table 2. There were eight experiments in the series. Four pairs of sartorius muscles and four pairs of gastrocnemius muscles were used. In respect to extent of shortening, the gastrocnemius muscles gave the more uniform results, as the table shows. All the fatigued gastrocnemius muscles shortened less than their non-fatigued mates—giving an average of 7.4 per cent less shortening in heat rigor. Checking this against the similar comparison where the whole process was carried out in air, it will be seen that the average difference of heat rigor shortening between fatigued and non-fatigued gastrocnemius mates was less than one-third as great in the atmosphere

TABLE 2

Comparison of extent of shortening in heat rigor of fatigued and non-fatigued muscles in an atmosphere of pure CO_2

(Both muscles placed in CO_2 after which one was fatigued and then both brought to heat rigor in the CO_2)

EXPERIMENT NUMBER	CONDITION OF MUSCLE	TIME USED IN FATIGUING PROCESS	TEMPERATURE DURING FATIGUING PROCESS	TEMPERATURE AT WHICH RIGOR BEGAN	DEGREES *a* BEGAN RIGOR LOWER THAN *b*	TEMPERATURE AT WHICH FIRST STEP OF RIGOR ENDED	EXTENT OF SHORTENING	PER CENT WHICH FATIGUED MUSCLE SHORTENED LESS THAN ITS MATE	COMPARATIVE POSITIONS OF *a* AND *b* MUSCLE LEVERS IN EARLY PART OF RIGOR
				Gastrocnemius muscle					
		minutes	*°C*	*°C*	*°C*	*°C*	*cm.*		
270	*a**	18	19.0	30.0	9.5	45.5+	5.3	11.6	At 39.5° *a* had risen 2.2 cm. and *b* was only beginning to rise
	b†			39.5		46.0	6.0		
272	*a*	17	19.0	29.0	9.0	44.5	5.5	9.8	At 39.5° *a* had risen 1.6 cm. and *b* only about 0.15 cm.
	b			38.0		46.0	6.1		
274	*a*	18	20.5	28.0	11.0	44.5	5.5	5.1	At 40°C. *a* had risen 2.2 cm. and *b* had only risen about 0.1 cm.
	b			39.0		45.0	5.8		
276	*a*	17	19.8	25.0+	12.0+	43.0	5.8	3.3	At 39° *a* had risen 2.7 cm., and *b* only 0.1 cm.
	b			38.0		44.8	6.0		
Average					10.4			7.4	

Sartorius muscle

269	*a**	10	24.0	36.0	2.0	43.0	10.1	4.7	At 40°C. *a* had risen 6.3 cm. and *b*, only 2.3 cm.
	b†			38.0		44.5	10.6		
271	*a*	9	19.5	34.5	2.5	43.0	9.7	3.0 *more*	At 37°C. *a* had risen 4.5 cm. and *b* was barely starting to rise
	b			37.0		44.5	9.4		
273	*a*	12	19.0	36.0	2.0	42.5	10.0	9.0 *more*	At 38°C. *a* had already risen 4 cm. and *b* not at all
	b			38.0		43.5	9.1		
275	*a*	7	19.0	35.0	2.0	42.0	8.3	5.1	At 37°C. *a* had already risen 2 cm.
	b			37.0		43.0	9.8		
Average					2.1			4.9 less (expts. 269, 275) 6.0 more (expts. 271, 273)	

Sartorius muscle
(In an atmosphere of pure nitrogen)

334	*a**	12	21.0	36.0	3.5	44.0	8.6	8.9	
	b†			39.5		46.0	10.0		

Gastrocnemius muscle

335	*a*	11	21.0	28.0	9.0	46.0	5.6	8.1	
	b			37.0		46.0	6.1		

* Fatigued.
† Non-fatigued.

of pure CO_2 as in pure air (i.e., 7.4 per cent as against 24.3 per cent less). On the other hand, there is a greater difference between the temperatures at which heat rigor began in the fatigued muscles and their non-fatigued mates, in the CO_2 series of experiments, than in the series of similar experiments carried out in air. Gastrocnemius muscles fatigued in CO_2 began shortening, on the average, 10.4°C. lower than that of their non-fatigued mates—the similar average temperature difference in air being only 8.7°C. in the author's experiments; 9.9°C. in Latimer's experiments.

Two of the sartorius muscles fatigued in CO_2 shortened a little less (average 4.9 per cent less), and the other two, a little more than their mates—but sartorius muscles fatigued in air shortened, on the average, 40 per cent less than their non-fatigued mates in heat rigor. It is clear, at least, that fatiguing sartorius muscles in CO_2 affected the extent to which they could shorten in heat rigor much less than when the fatiguing was done in air, just as was found true for gastrocnemius muscles. On the other hand, again, as was true for gastrocnemius muscles, the temperature difference at which rigor began is greater in the CO_2 than in the air experiments. Heat rigor started 2.1°C. lower (average) than in non-fatigued mates in case of sartorius muscles fatigued in CO_2, and 1.0°C. lower (average) when fatiguing was done in air.

In those experiments where opportunity was given for recovery after fatigue in pure oxygen, at low temperature, the recovery not only enabled the muscle to shorten, in heat rigor, more like a normal muscle, but it tended, also, to raise the temperature at which rigor began to more nearly that of the non-fatigued muscles. In the sartorius, restoration appeared to be perfect in this respect. With gastrocnemius muscles, when recovery had gone on in oxygen until the fatigued muscles shortened, on the average, 10.7 per cent (instead of 24.3 per cent) less than normal, the temperature at which rigor began in these muscles had been restored toward normal until they began rigor only 5°C. (instead of 8.7°C.), on the average, below the temperature at which rigor began in their normal mates. But, in the entire absence of oxygen, when fatigue in CO_2 had affected the gastrocnemius muscles until their average shortening in rigor was only 7.4 per cent less than normal, the average temperature at which they began rigor had been lowered the maximal amount, viz., 10.4°C. It will be observed from table 2 that standing in CO_2 for the period of the experiment did not materially change the temperature at which the non-fatigued muscles began heat rigor. It actually raised the average temperature of rigor a little more than 0.5°C. for the gastrocnemius muscles. Neither did *long* standing in CO_2 materially change the temperature at which rigor began in non-fatigued muscle. In one test experiment a non-fatigued muscle was kept in CO_2 for 22 hours and then it began heat rigor at 38°C.—which is but slightly above the average temperature of heat rigor in non-fatigued gastrocnemius muscles and really within the normal range.

TABLE 3

Effect of fatigue on extent of shortening of frog muscle in chloroform rigor (isotonic conditions)

EXPERIMENT NUMBER	CONDITION OF MUSCLE	TIME USED IN FATIGUING PROCESS	ROOM TEMPERATURE OF EXPERIMENT	WEIGHT OF MUSCLE AT BEGINNING OF EXPERIMENT	WEIGHT OF MUSCLE AFTER CHLOROFORM RIGOR	LOSS IN WEIGHT DURING CHLOROFORM RIGOR	EXTENT MUSCLE LEVER WAS LIFTED IN CHLOROFORM RIGOR	PER CENT THE FATIGUED MUSCLE SHORTENED LESS THAN ITS NON FATIGUED MATE
		minutes	*°C.*	*grams*	*grams*	*gram*	*minutes*	
Gastrocnemius muscle								
14	Normal						132	19.6
	Fatigued	30	18				106	
18	Normal						129	
	Fatigued	21	18				100	22.4
19	Normal			1.23	0.92	0.31	128	
	Fatigued	10	18	1.17	0.87	0.30	76	40.6
20	Normal			1.15	1.07	0.08	123	
	Fatigued	30		1.11	1.05	0.06	114	7.2
21	Normal			1.37	1.08	0.29	131	
	Fatigued	32		1.44	1.19	0.25	98	25.1
22	Normal			1.05	0.81	0.24	145	
	Fatigued	33		1.00	0.92	0.08	136	6.2
23	Normal			1.38	1.18	0.20	139	
	Fatigued	31	17	1.42	1.29	0.12	99	28.7
24	Normal			1.21	1.02	0.19	144	
	Fatigued	46	17	1.30	1.21	0.09	91	36.7
25	Normal			1.21	1.06	0.15	120	
	Fatigued	37		1.18	1.09	0.09	94	21.6
26	Normal			1.59	1.30	0.29	150	
	Fatigued	50	21	1.55	1.37	0.18	138	8.0
28	Normal			1.08	0.80	0.28	129	
	Fatigued	25	19	1.13	0.925	0.205	107	17.0
29	Normal			0.82	0.61	0.21	119	
	Fatigued	35		0.825	0.64	0.185	87	26.8
Sartorius muscle								
27	Normal						238	
	Fatigued	41	26				192	19.3
Average								21.4
Gastrocnemius muscle								
46	Normal			0.955	0.76	0.195	132	
	Fatigued	216	20	0.98	0.82	0.16	120	9.0

TABLE 4

Effect of fatigue on development of tension in frog muscle during chloroform rigor (isometric conditions)

Gastrocnemius muscle

EXPERIMENT NUMBER	CONDITION OF MUSCLE	TIME USED IN FATIGUING PROCESS	ROOM TEMPERATURE OF EXPERIMENT	WEIGHT OF MUSCLE AT BEGINNING OF EXPERIMENT	WEIGHT OF MUSCLE AFTER CHLOROFORM RIGOR	LOSS IN WEIGHT DURING CHLOROFORM RIGOR	HOURS AFTER RIGOR BEFORE WEIGHING MUSCLES	INITIAL TENSION USED IN EXPERIMENT	TENSION AT END OF CHLOROFORM RIGOR	PER CENT OF TENSION FATIGUED MUSCLE DEVELOPED LESS THAN NON-FATIGUED MATE	TENSION OF TETANUS
		minutes	*°C.*	*grams*	*grams*	*gram*	*hours*	*grams*	*grams*		*grams*
32	Normal	25	19	1.02	0.91	0.110	2	0	132.00	9.0	
	Fatigued			0.95	0.86	0.090		0	120.00		
33	Normal			0.99	0.91	0.080		7.0	104.00		
	Fatigued	38		0.99	0.94	0.050	2	7.0	92.00	11.5	
36	Normal			1.06	0.86	0.200		17.5	105.00		
	Fatigued	66	15	1.015	0.86	0.155	1½	17.5	94.00	10.4	
37	Normal			1.04	0.925	0.115		25.0	119.60		
	Fatigued	21		1.05	0.965	0.085	1½	25.0	103.50	13.4	282.0
38	Normal			0.67	0.54	0.130		31.5	185.64		
	Fatigued	32		0.67	0.575	0.095	1½	31.5	160.65	13.4	304.5
39	Normal			0.53	0.36	0.170		21.0	146.37		
	Fatigued	37		0.55	0.42	0.130	2¼	21.0	133.87	8.5	264.18
40	Normal			0.46	0.34	0.120		17.5	142.80		
	Fatigued	48		0.44	0.37	0.070	1½	17.5	132.09	7.5	249.1
41	Normal			0.56	0.415	0.145		45.0	182.07		
	Fatigued	73		0.565	0.44	0.125	15	45.0	171.36	5.8	285.6
42	Normal			1.28	1.13	0.150		21.9	167.80		
	Fatigued	140		1.33	1.20	0.130	¼	21.9	149.90	10.0	307.0
Average nos. 32 to 42										9.9	
44	Normal			0.92	0.77	0.150		28.0	135.60		
	Fatigued	93	20	1.00	0.78	0.220	1¼	28.0	82.10	39.0	274.8
45	Normal			1.205	0.83	0.375		15.8	182.00		
	Fatigued	100	18	1.24	0.94	0.300	2¼	15.8	92.80	49.0	253.4

As shown in table 2, two other experiments carried out in nitrogen (i.e., in exactly the same manner as those just described for CO_2), so as to exclude all oxygen, gave exactly similar results.

Effect of fatigue on extent of chloroform rigor-shortening of frog muscle. A series of sixteen experiments was run to obtain a comparison between the extent of shortening of unfatigued muscles in chloroform rigor and that of their fatigued mates under the same conditions. Through accident, three of these experiments failed. Results of the other thirteen experiments of the series are given in table 3. In every case, the non-fatigued muscle shortened more than its fatigued mate in chloroform rigor, just as in heat rigor. On the average the fatigued muscles shortened, in chloroform rigor, 21.4 per cent less than their unfatigued mates. The results for individual experiments were not so nearly uniform as in the case of heat rigor, and the average (21.4 per cent) is a little lower than the similar average of 24.3 per cent found in case of heat rigor.

Effect of fatigue on development of tension in chloroform rigor. The tendon of a muscle in chloroform rigor (unlike that of a muscle in heat rigor) remains quite strong; and the connective tissue of the tendon does not shorten in chloroform vapor. It was decided, therefore, to obtain isometric chloroform rigor curves of fatigued muscles and non-fatigued mates for comparison.

Two springs practically alike in length and strength were selected for the attachment of two isolated muscles (mates) so that comparable isometric contractions might be obtained. The experiments were carried out with the muscle chamber used in former experiments; so that the muscles were subject to the same conditions, except that one was fatigued by repeated stimulation while its mate was kept at rest. Results are given in table 4. The initial tension was varied in the different experiments but it was made the same for the two muscles (just as was temperature, etc.) in any individual experiment.

The temperature, in the different experiments, was never lower than 15°C. nor higher than 20°C., and, in each experiment, when the proper moment came, the muscles were enclosed in the same chamber of air saturated with chloroform vapor at the temperature of the experiment.

At the beginning of rigor, the fatigued muscles developed tension a little more rapidly than the non-fatigued, but were soon overtaken and surpassed by the latter, in every case, as rigor proceeded. This is taken to mean that the fatigued muscle cells were penetrated by chloroform and sent into rigor a little more readily than those of the non-fatigued muscle. At any rate, since rigor developed a little more rapidly, we may infer that more cells must have made a concerted pull on the lever, at first, in case of rigor of the fatigued muscles. And, other things being equal, the greater the number of cells that act together (in concert, rather than in tandem) the

greater the tension that should be developed isometrically. The fatigued muscles had the advantage over their mates, in this respect, during isometric chloroform rigor. In rigor under isotonic conditions, of course, where a small weight of only about two grams was lifted in every position of the lever, it would not make so much difference whether cells went into rigor in concert or, more slowly, a few at a time, so long as the cells going into rigor at any one time could readily lift the lever. Nevertheless, in spite of the advantage mentioned above, in the nine experiments (32 to 42), the fatigued muscles averaged 9.9 per cent less tension than their unfatigued mates, in chloroform rigor. This *tension* difference (9.9 per cent) is less than half the average percentage difference of *shortening* between fatigued muscles and their non-fatigued mates (21.4 per cent), as obtained from results of isotonic chloroform rigor shortening given in table 3.

Clearly the fatigued muscles were less capable than their mates in chloroform rigor contraction; and it is clear, also, that the difference in *isotonic* chloroform rigor contraction gives the truer picture of the magnitude of this difference in capability of the fatigued and non-fatigued muscle cells. However, for comparative purposes the isometric experiment results were the more uniform.

In experiments 44 and 45 a very strong stimulus was used for 93 minutes and 100 minutes, respectively, (continuing even after fatigue was complete) until some of the muscle cells had begun to die and slowly develop a slight rigor tension before chloroform rigor was started. Because of this *gradual* death and consequent rigor of many muscle cells, killed by continued strong stimulation, before the more rapid chloroform rigor was begun, large percentage differences in tension between fatigued and non-fatigued muscles were obtained—viz., 39 per cent where strong stimulation continued 93 minutes, and 49 per cent where the period was 100 minutes. In experiment 42 a much longer period of stimulation (140 minutes) had been used, but with a barely maximal stimulus so that no muscle cells were brought into rigor at all until chloroform was introduced. Note that the average percentage difference was obtained in that case. Finally, in experiment 46 one muscle was fatigued with *strong* stimuli, continued over a period of 216 minutes until a partial rigor had developed because of cells that had been killed (as in experiments 44 and 45). This muscle and its non-fatigued mate were then caused to pass into chloroform rigor under *isotonic* conditions. The results obtained, as shown in table 3, are not unlike those of other isotonic chloroform rigor experiments where the muscles were attached to very light levers. The findings of these four experiments, just related, confirm the explanation already given with respect to the discrepancy between average percentage *tension difference* and the average percentage *difference in shortening* of fatigued muscles and non-fatigued mates in chloroform rigor. Similarly, it may be observed

that tetanus tension of muscles was consistently higher than chloroform rigor tension of the same muscles, because all the cells can be thrown into a concerted contraction in tetanus, but the penetration of chloroform vapor into the muscle was not rapid enough to give an entirely concerted rigor contraction of all the cells. Of course, cells that have already gone into rigor are of no assistance to other cells of the muscle, going into rigor later, in building up any higher tension. On the other hand, chloroform rigor, under isotonic conditions, with a very light lever, usually gave a somewhat greater shortening than that of a tetanus contraction.

Loss in weight of the muscle during chloroform rigor. During chloroform rigor (either isotonic or isometric) there was a loss in the weight of the muscle. This appeared to be principally due to the fact that during the course of the development of the rigor contraction, and for a few minutes thereafter, drops of fluid exuded and dripped from the muscle. One reference to the fact that exudate is squeezed from a muscle, during chloroform rigor, was found in the literature, but quantitative results were not given. The drops of exudate began to appear in 10 to 15 minutes after the beginning of chloroform rigor. Under isometric conditions the maximum tension was reached within about 30 minutes with the gastrocnemius muscles used in these experiments; and the drip had entirely ceased within about 40 minutes. That exudate was squeezed from the muscle, for a few minutes after maximal chloroform rigor was attained, was made known also by the observation that it permitted the lever to decline slightly from the maximal height attained. Note that in experiment 41 where the muscles stood, under the full tension they had developed, for 15 hours after rigor was complete, there was no greater loss in weight (not quite so great a loss, in fact) than was given in experiment 39 where the muscles stood under their rigor tension for only $2\frac{1}{2}$ hours. The weights of the muscles, themselves, in the two experiments are comparable. Further, in experiment 42 the somewhat heavier muscles attained, proportionately, about the same loss in weight, although they stood only 15 minutes after rigor had developed its maximal tension. In other words, the drip (and the loss in weight) ceased within 10 to 15 minutes after attainment of the full rigor tension.

The 9.735 grams of unfatigued muscle that went into chloroform rigor under isometric conditions lost a total of 1.745 gram in weight—a loss of 0.179 gram for each gram of muscle. The 9.8 grams of fatigued muscle (mates), under the same treatment with chloroform vapor lost a total of 1.41 gram, or 0.143 gram, per gram of muscle used. That is, the non-fatigued muscles, per gram of muscle substance, lost 0.036 gram more than their fatigued mates in chloroform rigor.

Under isotonic conditions of chloroform rigor there was a loss of 0.185 gram for each gram of muscle used in the non-fatigued muscles (12.09

grams of normal muscle lost a total of 2.24 grams); and a 0.120 gram loss for each gram of muscle, in the fatigued mates (12.125 grams of muscle in chloroform rigor after fatigue lost 1.56 gram). Here, the non-fatigued muscles lost 0.065 gram more than the fatigued muscles, per gram of muscle used. The greater difference between loss of weight of non-fatigued and fatigued muscle in chloroform rigor occurred under isotonic conditions, therefore. One might expect that the normal, non-fatigued muscles would have lost more in weight (squeezed out more fluid as drip) in chloroform rigor under isometric than under isotonic conditions, but such was not the case, as the figures show.

SUMMARY

1. The general finding of Caroline W. Latimer (1898) that fatigued gastrocnemius muscles of frogs began heat rigor at a lower temperature and shortened much less than their unfatigued mates is confirmed, and the finding is extended also to sartorius muscles of frogs.

2. Fatigued, excised gastrocnemius muscles began heat rigor at an average temperature 8.7°C. lower than their non-fatigued mates, and shortened, on the average, 24.3 per cent less than their mates. Fatigued excised sartorius muscles began rigor at an average of 1.0°C. lower than their non-fatigued mates, and shortened, on the average, 40 per cent less than their mates. The comparisons are made for the first step in heat rigor shortening.

3. *Completely* fatigued excised gastrocnemius muscles, left standing in air (18° to 24°C.) showed no recovery, as indicated by any return toward the behavior of non-fatigued muscles, in heat rigor. If such mucsles were exposed to pure oxygen (16° to 18°C.) for suitable periods, however, a recovery of a little over 50 per cent in ability to shorten in heat rigor was indicated; and rigor began, then, on the average, at only 5°C. (instead of 8.7°C.) lower temperature than in case of the unfatigued mates. No recovery was indicated in oxygen at 25.5°C. Fatigued sartorius muscles, placed in oxygen, showed a little less than 50 per cent recovery in ability to shorten in heat rigor; however, they began shortening, thereafter, at practically the same temperature as their mates.

4. Both gastrocnemius and sartorius muscles were more quickly fatigued in either CO_2 or nitrogen than in air; but neither kind of muscle was affected so much, thereby, as regards heat rigor shortening as when fatigued in air. However, both showed a lower heat rigor temperature, on the average, than when fatigued in air.

5. On the average, fatigued muscles shortened 21.4 per cent less than their non-fatigued mates in chloroform rigor.

6. Fatigued muscles developed 9.9 per cent less tension in chloroform rigor than their non-fatigued mates.

7. Muscles going into chloroform rigor lost weight due to dripping of exudate squeezed from the muscle by the rigor contraction. The non-fatigued muscles dripped more exudate than their fatigued mates during rigor.

8. Suggestions as to why fatigued muscle behaves differently in heat rigor than does its non-fatigued mate are presented in an accompanying paper.

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EFFECT, ON HEAT RIGOR, OF TREATING SARTORIUS MUSCLES OF FROGS WITH RINGER'S SOLUTION CONTAINING SODIUM LACTATE OR CERTAIN OTHER SALTS; ALSO, WITH ACID AND ALKALINE RINGER'S SOLUTIONS

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1. Effect of sodium lactate, lactic acid, sodium hydroxide, hydrochloric acid, acid sodium phosphate and sodium bicarbonate. In order to test whether sodium lactate, applied from an outside source to muscle cells, would affect the temperature at which they would begin heat rigor, or their ability to shorten in heat rigor, the following method of experiment was used. Thin sartorius muscles (weighing 0.05 gram to 0.2 gram) were removed carefully from small frogs. It was found impractical to use a thicker muscle, like the gastrocnemius, because solutions penetrated too slowly to the deeper cells of such a muscle. Each muscle was attached to its lever in the apparatus already described (Shafer, 1928), and the levers carefully adjusted to the same base line under the same very slight tension (1 to 1.5 gram). One muscle was then surrounded by Ringer's solution (pH 8.4) and its mate by the same amount of Ringer's solution containing varying small amounts of sodium lactate. The muscles were allowed to stand thus at room temperature (18° to 22°C.) for different periods of time (2 to 18 hours). The solutions could be removed momentarily, if desired, and the muscles tested as to their response to electrical stimulus, or the muscles could be removed, weighed and replaced at any time. At the end of a determined period of treatment, the solutions were both removed, the muscles were enclosed, at once, in the same moist muscle chamber and the temperature gradually raised to cause heat rigor. The temperature at which rigor began in each muscle and the heights the levers were lifted in contraction were recorded. The sodium lactate used was obtained from Merck. It was acid in reaction so that when 2 per cent of the sodium lactate was placed in Ringer's solution of pH 8.4, the solution was changed to a pH of 5.2. When a sartorius muscle weighing 0.05 gram had been treated as described above with this acid sodium lactate Ringer's solution for only 25 minutes, it lifted its lever in contraction upon maximal single electrical stimulus only 67 mm., whereas the lever of its mate, that had been in Ringer's solution of pH 8.4, was lifted 96 mm. under the same

stimulation. After seven hours the solutions were removed again from around the muscles. The muscle which had been treated with acid sodium lactate Ringer's solution would not respond in the least to electrical stimulation now, while its mate, that had been treated during the same time, under the same conditions otherwise, in the ordinary Ringer's solution, responded normally to stimulation. The muscle chamber was placed around the two muscles and the temperature gradually raised to bring about heat rigor in moist air. The muscle that had been in Ringer's solution began rigor at 35.5°C. and completed the first step in rigor shortening at 46°C. Its mate showed no shortening whatever until a temperature of 54.5°C. was reached. That is, the treatment of the muscle (for the period of time given) with the acid sodium lactate Ringer's solution had abolished the first step in rigor shortening. The control muscle lifted its lever 83 mm. above the base line during the first step in rigor. When a temperature of 87°C. had been reached in the muscle chamber and the second and third steps in rigor in both muscles had been completed, the lever of the acid sodium lactate treated muscle stood 32 mm. above the base line and that of its normal mate 87 mm. above the base line.

In order to determine to what extent the acid condition and to what extent the sodium lactate was responsible for the effect on the muscle, as just described, two sets of experiments were then carried out, as follows. In the one set an excised sartorius muscle attached to its lever in the manner described above was treated with Ringer's solution brought to a certain pH (pH 3.2 to pH 5.4) with a slight amount of pure lactic acid and then compared in rigor with its mate which had been treated with ordinary Ringer's solution (pH 8.4) during the same time. In the other set of experiments one excised sartorius muscle was treated with Ringer's solution containing a certain percentage of sodium lactate (2 per cent to $\frac{3}{4}$ per cent sodium lactate was used in different trials) at a certain pH value. The pH of the acid sodium lactate in Ringer's solution was brought to any desired value by addition of standard NaOH before the experiment. In the latter series of experiments pH values were tried ranging from pH 5.4 to pH 9.8+. In every case the muscle treated with sodium lactate in Ringer's solution was compared in heat rigor with its mate which had been treated with ordinary Ringer's at pH 8.4 for the same time, and otherwise under the same conditions. Twenty-four experiments were run in this series in which sodium lactate was used, and sixteen experiments in the lactic acid set. Also eight experiments were carried out in which HCl (instead of lactic acid) was used as the acid in Ringer's solution to give it a pH of 5.2 to 5.4; and two experiments in which acid sodium phosphate (NaH_2PO_4) was used instead of lactic acid in the Ringer's solution. Space will not permit giving a protocol of each of these experiments, and they do not lend themselves to being condensed into a compact table of any kind.

But results of the different experiments justify the statement that treatment of the excised sartorius muscle of the frog with Ringer's solution containing the small amounts of the sodium lactate used, at any pH value tried, reduced the extent to which the muscle could shorten under electrical stimulation, and finally, the extent to which it could shorten in the first step of heat rigor. Furthermore, when a muscle had been treated for the right length of time (2 to 18 hours, varying with the acidity) with Ringer's solution rendered acid (pH 3.2 to 5.4) with any of the compounds just named, that muscle was found to require a *higher* temperature (1° to more than 20°C., higher temperature) than its mate to begin the heat rigor shortening. Most of the experiments gave temperature differences that were either small or large, but there were, also, intermediate differences giving a rise in rigor temperature of 3° and 5°C. When standard NaOH solution was added in small amounts to "Ringer's fluid containing the acid sodium lactate" (pH 5.4) until the pH of the solution was changed to about 9.4, the resulting solution no longer noticeably influenced the temperature at which the treated sartorius muscle began rigor, and it entered the first step of heat rigor at practically the same temperature as did its mate which had been in Ringer's solution only, at pH 8.4. If still more of the NaOH solution was added to the sodium lactate Ringer's solution until its pH was changed to 9.8 or a little more, then it caused the treated muscle to begin heat rigor at a *lower* temperature (1° to 4.5°C. lower, in this series) than its mate.

Sartorius muscles were now treated, until after they had been rendered unable to respond to electrical stimulation, with Ringer's solution containing *no* sodium lactate, but made alkaline to 9.8 or a little higher with sodium bicarbonate (ten experiments), and the rigor temperature was again found to have been lowered. This result suggested further experiments with other modified alkaline Ringer's solutions.

2. Effect of alkaline sodium phosphate and other hypertonic modified alkaline Ringer's solutions, on muscle, versus fatigue. Alkaline sodium phosphate (Na_2HPO_4) in modified Ringer's solution was used in a series of eighteen experiments carried out in the same way and gave substantially the same results as to the effects on sartorius muscle of frog as noted with the above alkaline Ringer's solutions. The phosphate Ringer's solution was made up as follows: NaCl, 6.5 grams; KCl, 0.14 gram; $CaCl_2$, 0.12 gram; Na_2HPO_4, 20 grams, and distilled water 1000 cc. When this solution was first made up it had a pH value around 9.4 or a little more. On standing in a cork stoppered bottle in air, the pH slowly changed to about 8.6, but even at the latter value it still brought about the same chain of effects on the muscle—although at the lower pH a longer time was required, and the temperature at which rigor started was not decidedly lowered. Of course, the pH of a phosphate solution in which a muscle was standing

gradually became lower, also. But 15 cc. to 18 cc. of freshly made, alkaline phosphate Ringer's solution around a sartorius muscle, weighing only 0.05 to 0.2 gram, sufficed to bring about the results. It was early noted that when an alkaline Ringer's solution of the right pH value was placed around a sartorius muscle attached to its lever, the muscle soon entered a more or less slow contraction, lifting the lever 2 to 8 mm., following which it slowly relaxed again; during this period it would respond to stimulus almost as well as its mate. After a still longer treatment its lever gradually sagged a little below the base line, and the muscle began to show a much diminished response to stimulus—and following that there appeared gradually the other effects of longer continued treatment that have been already described. After a good many experiments had been carried out, this initial slight slow contraction just described became recognized as a criterion for the most effective pH value of alkaline Ringer's solution to use in bringing about the series of effects on the muscle which have been pointed out, so closely like the effects of complete fatigue of the muscle upon its ability to respond to stimulus and upon heat rigor of the muscle.

Guenther (1905) and others have ascribed a similar slow contraction, obtained by treatment of frog's sartorius muscle with suitable solutions of KCl or $CaCl_2$ after the muscle had been in saline solution, to the influence of the cation. Whether, in the experiments described here, the contraction was due to changes in relative amounts of cations, or to the presence of certain concentrations of the anion (OH), it appeared to serve as a measure of correct alkalinity. If the solution was not alkaline enough to cause the initial small slow contraction at all, then it would not bring about the effect of finally causing the muscle to enter heat rigor at a lower temperature than its normal mate. If the alkalinity was too great, the muscle would be likely to enter a strong contraction in which it died without ever relaxing to the base line at all.

In connection with the experiments using the alkaline phosphate Ringer's solution, one other fact was learned which should be described here. It was found that if the freshly made alkaline phosphate Ringer's solution was allowed to act on the sartorius muscle over a long period of time (about 22 hours) then the treated muscle might require a much higher temperature (rather than a lower temperature) than its normal mate, to cause it to enter rigor. This higher temperature in fact was found to correspond to the temperature range of the next later step in rigor. A study of the entire series of alkaline treated muscles, in which treatment had been continued for longer and longer periods before heat rigor was induced, showed that after the alkaline treated muscles had been affected to the point when they began the first step in rigor shortening at a lower temperature than normal and shortened less, then, longer and longer periods of treatment affected the muscles so that they gave less and less extensive shortening during the

first step in heat rigor until, finally, after 18 to 22 hours of treatment, there was *no* shortening of the treated muscle at all during the period when its mate underwent the first step of heat rigor; and under this circumstance, the treated muscle began rigor only at the higher temperature range (54° to 62°C.). In other words, the first step in heat rigor shortening could be obliterated entirely by long continued treatment with the alkaline Ringer's solution, but during the first step of heat rigor, as long as there was any shortening whatever, it began at a lower temperature than normal. Now, the first step in rigor shortening of the muscle could be done away with entirely by long continued treatment with any of the acid Ringer's solutions described, as well as with the alkaline solutions. But one difference between the acid and the alkaline treated muscles stood out clearly. Whenever the treatment had been suitable to induce a temperature change at all, the acid treated muscle always required a higher temperature than normal to start the heat rigor contraction (i.e., even in the first step of rigor), while the first step of heat rigor began at a lower temperature than normal in the alkaline treated muscle, as long as it was still capable of shortening at all during that step.

Very gradual, slowly induced fatigue of an excised muscle (i.e., using slowly repeated electrical stimuli), brings about the now well known syndrome of effects: viz., a slight contracture first; then a gradually lessened response to electrical stimulus; finally, in complete fatigue, total inability to contract upon application of stimulus, and after that, susceptibility to enter heat rigor at a lower temperature, but with decidedly lessened ability to shorten during the first step in heat rigor—as compared with the normal unfatigued mate of the muscle. All this series of effects produced by fatigue could be duplicated by treating the excised muscle, for the proper length of time, with one of the alkaline Ringer's solutions of the proper pH value, as described above. Was the series of effects on muscle thus treated due, in fact, primarily to the alkalinity (at the proper pH) of the Ringer's solution used? Four experiments were tried in which NaOH was added to Ringer's solution to give about pH 10, and seven experiments in which $Ca(OH)_2$ was added to the Ringer's solution to give different pH values, from pH 9 to pH 9.8, in different experiments. In these eleven experiments the alkaline Ringer's fluid was seen to duplicate the entire series of effects, on the treated muscles, even to that of causing them to go into heat rigor at a lower temperature than their untreated mates. In three similar experiments where KOH was used and in four experiments where $NH_4(OH)$ was used to make the Ringer's solution show about pH 9, however, no definite result was obtained in the particular effect of lowering the temperature at which heat rigor began.

In all the foregoing experiments, in which muscles had been treated with fluids of different pH values, additions had been made to isotonic Ringer's

fluid—so that the resulting alkaline solution, with which the muscle was treated, was more or less hypertonic in every case. The alkaline sodium phosphate Ringer's solution, made up as described, was one of the more decidedly hypertonic solutions used. Its osmotic pressure was great enough to cause the muscle in it to lose weight during the first 2 to 3 hours. For example, a sartorius muscle weighing 0.145 gram placed in the alkaline phosphate Ringer's solution weighed only 0.12 gram after two and one half hours in the solution. If a muscle was left in the same solution some 6 to 8 hours, however, it gradually regained its weight and thereafter increased in weight in the solution. For example, a muscle weighing 0.15 gram at the beginning weighed 0.18+ gram after 15 hours in the solution. In this particular experiment the treated muscle lifted its lever only 4 mm. above the base line in the first step of heat rigor, as against 163 mm. for its mate (which had been kept for the same period in Ringer's solution pH 8.4). Indeed it was the regular thing to find that muscles which had been treated long enough with one of the alkaline Ringer's solutions to almost or quite do away with the first step of heat rigor shortening, had taken up water and increased in weight (the weight, of course, being taken just before heat was applied for heat rigor)—even though the treatment had been made with a somewhat hypertonic solution. Apparently the treating solution induced reactions in the muscle which changed the osmotic pressure of its protoplasm. Were these changes taking place after the death of the muscle cells or did they begin while the cells were to be considered as still alive?

3. Revival of muscle by calcium chloride. It was discovered that when a muscle had been treated with alkaline phosphate Ringer's solution until it had just reached the point where it would not respond to stimulus—even to a tetanizing stimulus (and for 20 to 30 minutes thereafter)—that muscle could still be revived, so that it would contract to some extent, by enclosing it for 20 minutes to 2 hours' time with a Ringer's solution containing excess calcium chloride. If the muscle was given a much longer treatment in excess calcium chloride Ringer's solution it again became irresponsive to stimuli. Ringer's solution containing M/32 calcium chloride was found to be best. After this soaking in excess calcium chloride Ringer's solution, the muscle would give a somewhat prolonged contraction at the point of stimulation, but, usually, the wave of contraction would not travel far beyond the point of stimulation, so that it was found necessary to move the tetanizing electrodes along the muscle from one end to the other in order to get the most pronounced contraction possible. Then several minutes might be required for complete relaxation. Certainly, then, the changes described all *began*, at least, before the muscle was dead. The muscles in these experiments were under only about 1.5 to 2 grams of tension. Ringer and Buxton (1887) found that they could strengthen and facilitate the

contraction of gastrocnemius muscles, weakened through trial stimulation and perfusion with 0.6 per cent saline solution, by the addition of phosphate of lime and calcium chloride to the perfusion solution. Likewise, Guenther (1905) obtained a restoration of the frog's sartorius by the addition of $CaCl_2$ to the saline used to immerse the muscle after it had been fatigued in 0.7 per cent NaCl solution. Following the above series, now, in several experiments, two sartorius muscles (mates), attached to their levers, were both treated with the same alkaline phosphate solution until they had just reached the point where they would not respond to tetanus stimulus. Then one of the muscles "A" was enclosed in excess calcium chloride (M/32) Ringer's solution, and the other, "B", in calcium-free Ringer's solution for a period of time—2 hours as a maximum. At the end of that time the solutions were removed, and it was found that the "A" muscle would respond to stimulus but the "B" muscle would not contract in the least. The muscle chamber was now placed around both muscles and heat rigor produced, in moist air, in the usual way. The "B" muscle began rigor, usually, at a little lower temperature than the "A" muscle. For example, in a typical experiment, "B" entered rigor at 32°C. and "A" at at 35.5°C. In these experiments the excess calcium and the calcium free Ringer's solutions were of the same pH value (i.e., 8.4) at the beginning. After having bathed the phosphate treated muscles, however, the calcium-free Ringer's solution was practically unchanged in every case while the excess calcium Ringer's solution had a lower pH—in two cases as low as pH 7.2. Some reaction of the phosphate treated muscle with the calcium containing solution had served to lower its pH value—and the muscle that had been in the calcium free Ringer's, which retained its pH unchanged and more alkaline, entered heat rigor at the lower temperature. In one further experiment, as in the last, the two sartorius muscles (mates) were treated for 6 hours, 37 minutes with a phosphate Ringer's solution (pH 8.9) until neither muscle would contract upon applying an ordinarily maximal electrical stimulus. "A" was then placed in excess $CaCl_2$ (M/32) Ringer's solution and "B" in calcium-free Ringer's solution. At the end of one hour the solutions were removed and the muscles tested by stimulus. "A" responded with a 5 mm. contraction, and "B" not at all. The solutions were replaced around the muscles as before, and then removed for testing the muscles again 25 minutes later. "A" gave a larger response, and now, by moving the tetanizing electrodes along the "A" muscle from one end to the other, a contraction resulted which lifted the lever 102 mm. as successive portions of the muscle came into contraction before the preceding portions had relaxed. This was the largest recovery contraction obtained in these experiments, and more than 15 minutes were required for complete relaxation of the muscle. The solutions were then replaced as before (excess $CaCl_2$ solution around "A" and calcium-free,

around "B") and left for thirteen and one-half hours longer. During this final treatment the muscle levers both gradually sagged a little below the base line—"B's" lever (10 mm.) about twice that of "A's" lever. With the solutions again removed, the muscles were tested by repeated stimuli as before. Neither muscle showed any contraction whatever. The moist air chamber was at once placed around the two muscles and heat applied for heat rigor. "B" began to shorten at 34.5°C. and "A" not until a temperature of 41.5°C. was reached. The solutions with which the muscles had been treated were now tested and the excess calcium solution gave pH 7.2; the calcium-free solution, pH 8.2. Longer treatment with these solutions had brought about a larger difference in the temperature at which rigor began. The muscle from the solution which had changed to the lower pH value required the higher temperature to start heat rigor shortening, although the pH of this solution was still on the alkaline side of neutrality—and the solution was slightly hypertonic. The calcium-free Ringer's solution was practically isotonic. The muscles weighed about 0.175 gram each at the beginning of the experiment. At the end of the 6 hours, 37 minutes in the alkaline phosphate Ringer's solution, they weighed 0.16 gram each (i.e., had not quite recovered their initial weight). At the end of the 15 hours in excess calcium Ringer's solution, just before heat rigor, "A" weighed 0.215 gram and "B" (taken from the calcium-free Ringer's) weighed 0.25 gram.

Evidently, differences in osmotic pressure were not primarily concerned in giving the result in question; but it was still by no means clear, in this case, that difference in pH value was the important factor. Indeed, eight experiments carried out with stronger excess calcium chloride (M/4) Ringer's solution made it clear that such a solution, at the pH (8.4) of ordinary Ringer's solution, could, in itself, rapidly affect sartorius muscles so that they would require a higher temperature than normal to start the heat rigor contraction. If one sartorius muscle was first fatigued, then it was more rapidly affected by the treatment with the excess $CaCl_2$ solution than was its mate which had not been fatigued. Usually the fatigued muscle gave a prompt and marked tonic contraction (1 cm. to 8 cm.) with slow relaxation when the excess $CaCl_2$ (M/4) Ringer's solution was first placed around it. The non-fatigued muscle lifted its lever only about 1 mm. (if at all) in such a tonic contraction.

4. Effect of modified alkaline Ringer's solutions made osmotically equivalent to ordinary Ringer's solution. It was decided to study the effect of alkaline Ringer's solutions on the sartorius muscle with osmotically equivalent solutions. Arrangements were made for four sets of experiments in which one of the muscles should be treated with a modified alkaline Ringer's solution that could be brought to any desired pH, above that of the regular Ringer's solution (pH 8.4) which was being used, but would always be

osmotically equal to it; the other muscle, to be treated for the same period of time with ordinary isotonic Ringer's solution,—i.e., isotonic to fresh muscle. (It should be mentioned that Ringer's solution which is isotonic to fresh muscle is not so to fatigued muscle—the latter showing a higher osmotic pressure.) In each set of experiments a different base was to be used to give the alkalinity; viz., (1) NaOH, (2) KOH, (3) $Ca(OH)_2$ and (4) NH_4OH.

To illustrate the general method used in making up one of the "modified alkaline Ringer's solutions" the specific case of no. 1 may be described. A solution was prepared containing electrolytes in the amounts given as follows: NaOH, 4.44 grams; KCl, 0.14 gram; $CaCl_2$, 0.12 gram; $NaHCO_3$, 0.20 gram and H_2O, 1000 cc. This gave an alkaline solution, osmotically equivalent to Ringer's solution, in which NaOH had been substituted in osmotically equivalent amount for the NaCl regularly present in Ringer's solution. In actual procedure a normal solution of NaOH was carefully prepared. Then the correct volume of this normal NaOH (i.e., to contain the 4.44 grams of NaOH)was measured accurately and diluted to 1000 cc. This NaOH solution was equivalent in osmotic pressure to a salt solution containing 6.5 grams of NaCl to 1000 cc. To the 1000 cc. volume of NaOH solution, then, the correct weights of the other electrolytes used in Ringer's solution for frogs, as described above, were added. The resulting "osmotically equivalent alkaline Ringer's solution" was of course quite alkaline—too alkaline to use on living muscle tissue. It could be added in small amounts, however, to ordinary Ringer's solution to give a modified alkaline Ringer's solution of any desired pH above 8.4 without change of osmotic pressure. A measured amount of ordinary Ringer's solution, say 50 cc., was taken and into this the osmotically equivalent alkaline Ringer's solution was accurately measured—drop by drop, toward the end—tests being frequently made on sartorius muscles attached to levers, until the alkalinity of the treating solution became such that the treated muscles gave the slow tonic contraction and relaxation (2 to 10 mm. at the writing point of the lever) which has already been described as the criterion for the apparent optimum alkalinity to use in obtaining the syndrome of effects described as similar to fatigue effects on muscle. From these proportions of ordinary Ringer's solution and osmotically equivalent NaOH Ringer's solution, a stock isotonic modified alkaline (NaOH) Ringer's solution could be prepared. By the same general method a stock of modified alkaline Ringer's solution, which was isotonic, was made with each of the other hydroxides mentioned. Just as NaOH stood in the place of a certain amount of NaCl in the final stock solution of no. 1 so, in case of no. 2, KOH replaced some of the NaCl; in no. 3, $Ca(OH)_2$ replaced some of the NaCl; and in no. 4, NH_4OH replaced some of the NaCl regularly present in Ringer's solution. Thus, the four different stock solutions used

in the experiments which followed, had different proportionate amounts of the cations regularly present in Ringer's solution; only one stock solution had a different cation (NH_4) introduced. All the solutions were alike in being isotonic with ordinary Ringer's solution, and in being more alkaline than it. Twenty-two experiments in all were carried out using the four stock solutions. In spite of the differences in relative amounts of cations present in the stock alkaline Ringer's solutions, the $Ca(OH)_2$, NaOH, and KOH solutions all gave the series of effects, on the treated muscles, comparable to the effects of fatigue; heat rigor of the treated muscle regularly beginning at a lower temperature than in its mate which had been in Ringer's solution (pH 8.4) up to the time of heat rigor. This was true unless the treatment with the alkaline solution had been carried on so long as entirely to do away with the first step of rigor, in which case the alkaline treated muscle required a higher temperature before any shortening of the muscle began, just as was found in the similar circumstance when alkaline sodium phosphate Ringer's solution was used.

In those experiments with the stock solution which had been made alkaline by $NH_4(OH)$, the results were hardly more decisive than in the preliminary experiments already reported. On that account experiments were repeated with that stock solution until nine experiments were included in the set. In these nine experiments the alkaline treated muscles began rigor at a lower temperature than their mates in four; at exactly the same temperature in two; and they required a little higher temperature in the other three experiments. The average temperature for the nine treated muscles was lower than the average for their untreated mates; but the difference was not great, as will soon appear. The average temperatures at which the treated and the untreated muscles went into heat rigor, in each of the four sets of experiments, fall into an interesting series as follows: average temperature at which the $Ca(OH)_2$ Ringer's treated muscles went into heat rigor, 28.8°C.; NaOH, 32.9°C.; KOH, 34.1°C.; $NH_4(OH)$, 34.4°C.; and the average temperature at which all the untreated muscles (mates) went into heat rigor, 35.0°C. Using that isotonic modified alkaline Ringer's solution (stock) that appeared to be at the optimum alkalinity in case of each of the four kinds of stock solutions, the gram molecules of the hydroxide per 1000 cc., that had actually been put into each one, was figured and found to be as follows: NaOH, 0.00647; KOH, 0.006475; $Ca(OH)_2$, 0.00219; and $NH_4(OH)$, 0.00323 gram molecules per liter. As determined by the hydrogen electrode, these same stock solutions gave the following pH values;[1] NaOH stock Ringer's solution, pH 9.61; KOH stock Ringer's solution, pH 10.98; $Ca(OH)_2$ stock Ringer's solution, 9.27; and $NH_4(OH)$ stock Ringer's solution, pH 9.54.

[1] The following four determinations with the hydrogen electrode were kindly made for me by Dr. J. P. Baumberger.

5. Comparison of the effects of calcium hydroxide and certain salts on the initial temperature of heat rigor. It will be noticed that the stock $Ca(OH)_2$ alkaline Ringer's solution was not the one which showed the greatest pH, or which contained the greatest gram molecular weight of hydroxide per liter, and yet it was the most efficient solution of the four, in the matter of lowering the initial temperature for heat rigor in the muscle. The stock $NH_4(OH)$ alkaline Ringer's solution contained a low gram molecular weight of hydroxide also (pH 9.54, however), but the NH_4OH could not be used in larger amount without causing extreme tonic contracture of the muscle. Apparently, the ammonia itself was showing a poisonous effect, in larger amounts.

Slight excess of $CaCl_2$ in the Ringer's solution had been observed to antagonize, in some way, the action of an alkaline solution on the muscle. It was decided to make further trial of this effect of $CaCl_2$, first in connection with the $NH_4(OH)$ Ringer's solution. When an excess $CaCl_2$ (M/16) Ringer's solution was used (instead of ordinary Ringer's) it was found necessary to add about three times the amount of the $NH_4(OH)$ Ringer's solution before the mixture would cause the slight tonic contraction and relaxation of sartorius muscle, i.e., the criterion contraction. A very slight flocculent white precipitate, undoubtedly carrying some of the calcium, separated, and was filtered out, after adding the $NH_4(OH)$. After a muscle had been treated with this filtered excess $CaCl_2$–$NH_4(OH)$ Ringer's solution for about $3\frac{3}{4}$ hours, until after a short tetanus stimulation would give only a 2 mm. contraction, it then required a higher temperature than normal (rather than a lower) to initiate the heat rigor contraction—e.g., 39°C., whereas the normal mate began rigor at 36°C.

In the next experiments excess $CaCl_2$ (M/16) Ringer's solution was made alkaline with osmotically equivalent $Ca(OH)_2$ Ringer's solution and the resulting mixture used to treat sartorius muscles. Again it was found that a larger amount of the hydroxide was necessary than when ordinary Ringer's (instead of excess $CaCl_2$ Ringer's) was used in making up the treating solution. In this case about twice the amount of osmotically equivalent $Ca(OH)_2$ Ringer's, as was used in former experiments with ordinary Ringer's solution, had to be added before the excess $CaCl_2$–$Ca(OH)_2$ Ringer's solution, resulting from the mixture, would give the tonic (criterion) contraction and relaxation. Again, after the alkaline Ringer's and the excess $CaCl_2$ Ringer's solutions were mixed, a very faint cloudy precipitate appeared; disregarding the slight loss of calcium that may have been occasioned thereby, the concentration of calcium in this mixed solution was M/18. After about 2 hours in this solution, when the treated muscles (two experiments) barely showed contraction on stimulation, heat was applied. In one experiment rigor began at 41°C. in the treated muscle and at 36°C. in the untreated mate; in the other experiment, at

41°C. in the treated muscle and at 37°C. in the mate which had been in Ringer's solution. The first step in rigor was completed in the untreated muscles at about 45°C. These latter experiments had been carried out with muscles from a new lot of frogs, in the spring of the year. The sartorius muscles from these frogs could be quickly fatigued with comparatively few contractions. In order to make sure that the difference in effect, of the excess $CaCl_2$ Ringer's solution, made alkaline with $Ca(OH)_2$, in making necessary a *higher* temperature to start the heat rigor contraction (rather than a lower temperature, as had been found in former experiments with ordinary Ringer's solution made alkaline with $Ca(OH)_2$) was really due to the presence of the excess $CaCl_2$, and not to some difference in the frogs themselves, trial was made now with sartorius muscles from frogs of this lot, using the stock mixture of ordinary Ringer's solution made alkaline with $Ca(OH)_2$; the treated muscle began heat rigor at the markedly lower temperature than its mate, just as in case of the earlier experiments. The excess $CaCl_2$ was undoubtedly causing the difference. Lillie (1909) had told of the ability of $MgCl_2$ to protect Arenicola larvae against the effects of either weak acid or alkali solutions. But in this case, with sartorius muscle, we have a neutral salt ($CaCl_2$) which could be placed at such concentration in Ringer's solution as to affect the muscle in the same manner as has already been described for an acid Ringer's solution; and, moreover, it could be shown to assert that effect even when placed in an alkaline Ringer's solution. Further, it was found that if a completely fatigued muscle was first treated for a short time with excess $CaCl_2$ (M/4) Ringer's solution (pH 8.4), before heat was applied, it would then require a higher temperature than normal for heat rigor. That is, the $CaCl_2$ in Ringer's, at the pH of ordinary Ringer's solution, could reverse the effect of fatigue (or the effect of alkaline $Ca(OH)_2$ Ringer's), with respect to the temperature required for rigor, and an increase of 5°C. above normal—i.e., a large increase within the temperature range of the first step of rigor—seemed to be easier to obtain with this excess $CaCl_2$ Ringer's than with acid Ringer's solution.

An excess $NaCl_2$ (M/8) Ringer's was now prepared and made alkaline with the $Ca(OH)_2$ Ringer's solution until, by trial, the mixture was found to give the criterion contraction when placed around a sartorius muscle. This mixed solution contained about M/200 calcium. The mate was kept in Ringer's solution, while the other muscle was treated for 1½ hours with the $NaCl_2$ (M/8)–$Ca(OH)_2$ Ringer's solution until it barely responded on short tetanus stimulation. The muscle was weighed at this time and was found to have made a slight increase in weight in spite of the fact that the treating solution was hypertonic. Before treatment the muscle weighed 0.12 gram and after the 1½ hours of treatment, 0.14 gram. The two muscles were then brought to heat rigor in the same moist air chamber. The

treated muscle began rigor at between 29° and 30°C.; the muscle that had been in Ringer's solution began rigor at 36°C. This showed clearly that NaCl, present in excess (so as to give as high concentration of the chlorine ion as was present in the excess $Ca(Cl)_2$–(M/16) $CaOH_2$ Ringer's solution used) did not counteract the usual effect of the alkaline $Ca(OH)_2$ Ringer's solution on the heat rigor temperature of the muscle.

Next, an excess calcium Ringer's solution was prepared with calcium lactate (M/32), the lactate being substituted in equivalent amount for a part of the NaCl of Ringer's solution. A sartorius muscle was tested with this excess $Ca(C_3H_5O_3)_2$(M/32) Ringer's solution made alkaline with $Ca(OH)_2$ until the criterion contraction was obtained. It was then treated further until it would not respond to stimulus; after which, the treated muscle and its untreated mate were brought to heat rigor. It began heat rigor at practically the same temperature as its untreated mate. An exactly similar experiment was made using (M/24) $Ca(C_3H_5O_3)_2$ in $Ca(OH)_2$ Ringer's. The concentration of the calcium in this mixed solution was about M/25. This was about as great concentration of the lactate as could be used and still have the solution isotonic—all but 2.85 grams of the NaCl per 1000 cc. having been substituted by its molecular equivalent of $(C_3H_5O_3)_2$; viz., 10.23 grams of the lactate per 1000 cc. were used. Again the treated muscle began rigor at practically the same temperature as its mate. In these two experiments the calcium salt did not entirely reverse the effect of the alkaline $Ca(OH)_2$ Ringer's solution, but it did prevent the alkaline solution from causing the muscle to begin rigor at a *lower* temperature than normal. In the last experiment, treatment was carried so far that the muscle could shorten only 4 mm. in the first step of heat rigor, but, even so, its rigor began at 36°+C. while that of its mate began at 36°C.

Finally, a series of experiments was arranged in which the osmotically equivalent $Ca(OH)_2$ Ringer's already described was added, in larger and larger amounts, to ordinary Ringer's solution; and the resultant alkaline Ringer's solutions were used to treat sartorius muscles. The object of this series was to see whether the solutions containing higher concentrations of $Ca(OH)_2$ would still cause the muscle to enter heat rigor at a lower temperature than normal, or whether the cation, calcium, when present in greater concentration, as the hydroxide, might then cause a reversal of the effect already noted for the $Ca(OH)_2$ Ringer's solution and so cause the muscle to require a higher temperature than normal before it would begin heat rigor. The different percentages of the osmotically equivalent $Ca(OH)_2$ Ringer's placed in the mixture with ordinary Ringer's solution in this series are as follows: 9 per cent, 12 per cent, 23 per cent, 53 per cent, 66 per cent and 100 per cent. The 100 per cent osmotically equivalent $Ca(OH)_2$ Ringer's solution, alone, contained 1.79 gram of $Ca(OH)_2$ per 1000 cc.—the $Ca(OH)_2$ replacing a part (i.e., its molecular equivalent) of

the NaCl in Ringer's solution. Its concentration of calcium was about M/40. The muscles treated with the first three mixed solutions of the series all began rigor at distinctly lower temperatures (average 6°C. lower) than their mates which had been in Ringer's solution only. The muscles treated with the three more alkaline solutions began rigor at practically the same temperatures as their mates. In the 66 per cent and the 100 per cent solutions the muscles began passing at once into extreme sustained contraction, and it appeared that the concentration of $Ca(OH)_2$ in these solutions was great enough to kill the cells about as fast as it was penetrating the muscle. The 100 per cent solution rendered the small sartorius muscle incapable of responding to stimulus at the end of 10 minutes—and in that short time the muscle had increased 40 per cent in weight. In the first step of heat rigor, which was brought on as quickly as possible after the 10 minutes of treatment, the muscle shortened only 17 mm. above the first strong sustained contraction already mentioned. In other words, with these two higher concentrations of the $Ca(OH)_2$, the treated muscles seemed to have few if any cells, at any one time, which were either not already dead from the treatment or else not yet influenced sufficiently by the $Ca(OH)_2$ to show an effect on the rigor temperature. On the other hand, in the case of that combination containing 9 per cent of the osmotically equivalent Ringer's solution (giving pH 9.27) the concentration of the $Ca(OH)_2$ appeared to be at such an optimum as to allow time for all the cells of the muscle to come under its influence before very many of them had been killed, or had been otherwise rendered incapable of showing the first step of heat rigor. Under this condition the greatest lowering of the heat rigor temperature was obtained. The solution, in this case, contained about M/312 calcium.

The anion, (OH), in the solutions used, seemed to be the essential factor necessary to bring about the lowered heat rigor temperature in the muscle, but it could not show itself equally effective when carried into the solution by different cations. Calcium seemed to be the most important cation in that connection.

When $CaCl_2$ and NaCl were compared, in the experiments, with respect to ability to raise the heat rigor temperature of sartorius muscle, the calcium ion seemed to be the essential factor rather than the anion. It is true that it seemed to be not so potent in this respect when it was present in the form of $Ca(C_3H_5O_3)_2$, mainly, as when introduced as $CaCl_2$, but the calcium was actually present in a little greater concentration in the $CaCl_2$ experiments. Moreover it is probable that the $Ca(C_3H_5O_3)_2$ ionizes less than $CaCl_2$ in Ringer's solution.

6. General discussion; and further evidence on the effect of carbonic acid as modified by anoxemia. The close similarity between the effect of fatigue and the effect of alkaline Ringer's solution (especially $Ca(OH)_2$ Ringer's

solution) on the sartorius muscle has been pointed out. It was pointed out, also, that when the muscle was placed in an alkaline Ringer's solution, it began to show increase in weight soon after other effects of the solution of the muscle became evident. Even when the alkaline Ringer's solution was somewhat hypertonic (as for example when Na_2HPO_4 was added to Ringer's solution) so that it had a higher osmotic pressure than the muscle, and caused the latter to lose weight at first, this weight was gradually regained while the muscle was in the solution, and thereafter the muscle took up water, increasing in weight and size. This showed that there had been a movement of ions through the cell membranes of the muscle which caused changes in the protoplasm such as to appear to raise its osmotic pressure. It is safe to assume that the other effects on the muscle are also largely due to the same cause; namely, to changes in the protoplasm resulting from the movement of ions through the cell membrane of the muscle. The changes in the cell resulting from fatigue induced by repeated stimulation certainly caused the same ultimate effects on heat rigor of the muscle as the changes in the cell resulting from the movement of ions through the cell membrane when induced by the presence of the alkaline $Ca(OH)_2$ Ringer's solution. The changes, themselves, may be identical in the two cases, but they are not necessarily so.

Many of the findings in this connection are hard or, with our present knowledge, seemingly impossible to reconcile.

For example, it is established by the observations of many workers that ordinarily the fatigued muscle is acid while the non-fatigued muscle is slightly alkaline in reaction. If hydrogen ion or hydroxyl ion concentration, as such, were the determining factor of the effect of fatigue on heat rigor of muscle, one might expect the experiments with acid and alkaline Ringer's solutions around the muscle to give exactly the opposite effects to those they are found to give in the matter of changing the temperature of heat rigor.

Hoet and Kerridge (1926) have confirmed the early observation of Bernard that a rapid onset of rigor, associated with alkaline reaction, was to be found in crustacean muscles poor in glycogen. It is true that the fatigued muscle is poorer in glycogen than its non-fatigued mate; but the muscle may still have a considerable store of glycogen after complete fatigue, and yet be so changed in the condition of fatigue as to go into heat rigor at the lower temperature, or enter death rigor more promptly than its unfatigued mate, and apparently show an acid reaction at the same time.

The fact that excess $CaCl_2$ (M/4) Ringer's solution gave the same effects upon muscle as did acid HCl Ringer's solution—both being opposite, in their effects, to those of alkaline Ringer's solution on the muscle, should be mentioned now in connection with the finding of Embden (1923) that

M/32 $CaCl_2$ is most efficient in the synthesis of lactacidogen while M/4 $CaCl_2$ breaks it down. Here, it is not clear why M/4 $CaCl_2$ should give the effects on muscle similar to those of acid Ringer's solution if, as Embden states, it breaks down lactacidogen—thus showing an activity which alkaline solutions are known to have.

Latimer (1899) concluded that the loss of glycogen in the fatigued muscle was responsible for the difference in heat rigor contraction and the lower temperature of rigor as between the fatigued muscle and its non-fatigued mate. But the lowered rigor temperature can not be due to difference in glycogen content, merely, since the author, Shafer (1928), found that after fatiguing the muscles in pure CO_2 with less than one-third of the number of contractions as in air (and therefore with the consumption of much less glycogen) the maximal lowering of heat rigor was nevertheless obtained.

Again, Meyerhof (1921) found that with dibasic alkali phosphate (Na_2HPO_4 or K_2HPO_4) of suitable concentration in minced muscle, all the glycogen could be changed to lactic acid. But in two preliminary experiments, Dr. Laurence Irving kindly determined for me that glycogen was still present, in excised sartorius frog muscles, in considerable amount after they had been treated with alkaline $Ca(OH)_2$ Ringer's solution (pH 9.3), in the one experiment, and in the other experiment, with acid HCl Ringer's solution (pH 5.2) until they would show no shortening in the first step of heat rigor. In the muscles treated with acid Ringer's solution, he found no inorganic phosphates present; i.e., none soluble in trichloracetic acid. In the HCl Ringer's solution with which these muscles had been treated, however, he found inorganic phosphates present in amount equal to that ordinarily present in frog muscle. Large numbers of frogs were necessary to carry out the determinations accurately, but it is hoped that further work along this line may be carried on later.

Hartree and Hill (1923) and likewise de Nito, Oberzimmer and Wacker (1924) have shown that alkali proteins are present in living protoplasm, which must certainly have a part in neutralizing acids during the process of fatigue and during rigor. Fisk and Subbarow (1928) have pointed out the presence of phospho-creatin as a buffer in muscle. From this we must conclude that considerable of the bases held in combination with proteins and phospho-creatin, in fresh muscle, exist, in fatigued muscle, in the form of lactates, and primary and secondary phosphates. Furthermore, if the immediate lactic acid precursor (lactacidogen) is hexosediphosphate (intermediate between glycogen and lactic acid) then, especially, would a larger amount of the phosphoric acid be present as inorganic phosphates in the fatigued muscle—and this would be true of the muscle treated with alkaline Ringer's solution as well.

Experiments have been described (Shafer, 1928), which showed that

pure CO_2 gas around the muscle could not keep the fatigued muscle from going into rigor at the lower temperature; lactic acid or hydrochloric acid (pH 5.2) in Ringer's solution *could* do that, and moreover either of the latter acids could so change the fatigued muscle that it would require a higher temperature than normal for heat rigor. Now, when pure CO_2 is around the moist living muscle, we must suppose, from the work of Jacobs (1920) as well as from the work of Brooks (1923), that it saturates the muscle tissue fluid and that the concentration of carbonic acid, as such, is high in the muscle. Up to this point in the investigation, therefore, it seemed inexplicable that acidification of the muscle with carbonic acid should give a different result in this respect than acidification with HCl or lactic acid, but it was true that pure CO_2 gas around the muscle did not prevent the lower rigor temperature in muscle after fatigue. As will be observed, however, the CO_2 had not been applied in solution in Ringer's fluid around the muscle as was done in using the other acids.

The following experiments were carried out to determine whether carbonic acid applied to the muscle in Ringer's solution would give a similar result to the other acids.

One sartorius muscle was fatigued completely in air and then surrounded with Ringer's fluid through which a fine stream of CO_2 was bubbling to give the solution a pH of about 5.4. The unfatigued mate of this muscle was surrounded by ordinary Ringer's solution (pH 8.4), during the same time. The treatment of the muscles lasted for different periods of time in different experiments—from $\frac{1}{2}$ hour to 6 hours. Seven experiments were tried. The Ringer's solution, acid with carbonic acid, rapidly diminished the ability of the muscle to shorten in the first step of rigor. In two of the experiments treatment with CO_2 Ringer's solution (for 6 hours and 2 hours, respectively) was carried so far that the muscles showed no shortening at all in the first step of rigor. In the other five experiments (where treatment lasted for periods between 1 hour and $\frac{1}{2}$ hour, inclusive) the muscles treated with CO_2 Ringer's solution began heat rigor at an average of 0.6°C. higher than the unfatigued mates that had been for the same periods in Ringer's solution. This indicated that the carbonic acid Ringer's solution had influenced the fatigued muscles in the same direction as had been found for other acids and for excess $CaCl_2$ in Ringer's solution, although the difference was not very striking.

Eight experiments were then tried in which neither sartorius muscle, of a pair, was fatigued but one was treated for a definite period with CO_2 Ringer's solution—the carbonic acid giving the solution a pH of 5.4—as in the last experiments, and the mates were treated for the same period, in each case, in Ringer's solution of pH 8.4. In three of these experiments the muscles were treated with the acid Ringer's solution too long ($15\frac{1}{2}$ hours, $10\frac{1}{2}$ hours and 8 hours, respectively), i.e., until they did not shorten

at all in heat rigor. In the other five experiments the muscles treated with CO_2 Ringer's solution began heat rigor at an average temperature 3.8°C. *lower* than that of their mates, rather than higher. How could these results be reconciled? Muscles treated with Ringer's solution made acid with HCl, et al., (and containing air in solution) never began rigor at a lower temperature than their normal mates.

Five experiments were now tried in which one muscle was treated with Ringer's solution through which nitrogen gas bubbled to exclude all oxygen, and the mate was kept in Ringer's solution containing oxygen from air; both solutions being at pH 8.4. The time of treatment varied from ½ hour to 22 hours. In all these experiments the muscle which had been kept in a state of anoxemia (i.e., in nitrogen Ringer's solution) began heat rigor at a lower temperature than its mate—a treatment of ½ hour gave 1.5°C. lower, and 22 hours' treatment gave 7°C. lower rigor temperature. The average heat rigor temperature was 4.3°C. lower for the muscles subject to anoxemia.

From this last result it became clear that the fatigued muscles treated with CO_2 Ringer's solution were really subject to two opposite influences—the one a pH (acid) influence, tending to raise rigor temperature, and the other a result of anoxemia, tending to lower the rigor temperature. The HCl Ringer's solution which had been used on fatigued muscles, on the other hand, contained oxygen, from the air; these muscles, therefore, had been subject to two influences, *both* of which tended to raise the rigor temperature; viz., the pH (acid) and the "oxygen recovery" (due to the presence of oxygen) influences. It has been shown by the author (1928) that oxygen recovery raised the rigor temperature of a fatigued muscle.

The effect of carbonic acid in Ringer's solution (pH 5.4) on the heat rigor temperature of fatigued muscle was, therefore, partly masked because the Ringer's solution, saturated with CO_2, could not be applied without, at the same time, imposing anoxemia on the muscle. Otherwise, the effect of CO_2 Ringer's solution on the heat rigor temperature was the same as that of HCl Ringer's solution at the same pH. Also, the "carbonic acid Ringer's solution" at the same pH (5.4) was as effective as the HCl Ringer's solution in diminishing the ability of sartorius muscle to shorten during the first step of rigor, and could gradually render the muscle incapable of shortening at all during the first step of rigor.

In three experiments, exactly the same modified excess $CaCl_2$ (M/16)–$Ca(OH)_2$ Ringer's solution (pH 9.3) was used around one sartorius muscle (*a*) as had been used in the former experiments (page 160), but under the different condition that nitrogen gas was kept bubbling through the solution constantly (beginning 15 minutes before the solution was placed around the muscle) to exclude all oxygen. The mate (*b*) was kept during the experiment period in ordinary Ringer's solution (pH 8.4) containing air in

solution. In two of these experiments heat rigor began in the (*a*) muscle at exactly the same temperature as in the (*b*) or normal muscle. In the third experiment, treatment was carried so far that the (*a*) muscle shortened less than 1 mm. in the first step of rigor, and the *exact* rigor temperature could therefore not be determined, but it was very close to that at which its mate began heat rigor. In other words, the $CaCl_2$ could not show its *marked* effect of raising the temperature of heat rigor in the absence of oxygen—a result which seems to suggest that $CaCl_2$ favors oxidation recovery in the muscle; just as, also, the indication seems to be that an acidity of the muscle, slightly above the normal, may favor oxidation recovery. Directly in line with this first suggestion is the finding of Martin (1906) with respect to heart muscle, that "the chief function of calcium is to promote oxygen absorption" by the heart tissue, since the usual calcium effects on heart muscle were not obtained in the absence of oxygen. He was using $CaCl_2$.

Finally then, the statement seems to be justified that "oxidation recovery" and slight acidity (pH 5.4) as well as excess $CaCl_2$, in the presence of oxygen, are all alike in their effect of tending to raise the rigor temperature of frog's sartorius muscle, while fatigue (especially under conditions of oxygen deficiency for the muscle) and slight alkalinity (about pH 9.2) both tend to the opposite effect of lowering the rigor temperature.

It was true that fresh unfatigued sartorius muscle could be kept for as long as 22 hours in pure CO_2 gas without showing very great diminution in its ability to shorten during the first step of rigor, while, as has just been pointed out, CO_2 Ringer's solution progressively reduced the ability of the muscle to shorten in heat rigor (just as did HCl Ringer's solution) until, after some hours of treatment, the muscle showed no shortening whatever during the first step of heat rigor of its normal mate. Of course, muscle tissue fluid is a better buffer than Ringer's solution, but in spite of that, muscle tissue fluid in pure CO_2 gas must become acid—so that the results seem contradictory with respect to effect on the ability of the muscle to shorten in heat rigor. However, in the one case the only fluid around the muscle cells was the extremely *limited* volume of tissue fluid, acid with carbonic acid; in the other case, the muscle was surrounded by a comparatively *large* volume of acid Ringer's solution. It is likely that acid tissue fluid, because of its extremely limited amount, can dissolve only an insignificant amount of acid soluble materials from the cells before it becomes saturated, while the comparatively very large volume of acid Ringer's solution may gradually remove almost all such materials from the muscle cells. Significant here is Irving's finding (page 165) that all the phosphates had been extracted and were present in the HCl Ringer's solution with which muscles had been treated, while glycogen was still plainly present in the muscle; treatment, in that case, having been carried to the point where the muscles could show no shortening in heat rigor.

All these findings, then, seem to point to the following suggestion; viz., that whether the muscle is treated with Ringer's solution slightly more alkaline than usual (as pH 9.6) or is fatigued by repeated stimulation, it may very well be the higher than ordinary concentration of inorganic phosphate in the muscle (brought about by the breakdown of hexose phosphate, etc.), under conditions that finally inhibit its resynthesis by oxidation, that is responsible for the lowered temperature of heat rigor.

Hoet and Marks (1926) conclude, after careful analysis of their findings, that "absence of glycogen and the decrease in lactacidogen of the muscle are the determining factors" of the precipitate rigor observed in rabbits dying after hypoglycemic convulsions or after prolonged thyroid feeding. Under the conditions of the experiments described in this paper, we have seen that it is not necessary to have an entire absence of glycogen to get the lowered rigor temperature. Of course, this lowered critical temperature of heat rigor would tend to give the precipitate or rapid onset of rigor in case of a mammal at *its usual body temperature*. Again, the suggestion is that a comparatively high concentration of inorganic phosphates in the muscles (occasioned by the breakdown of some of the glycogen or other lactic acid precursor, in the muscle, either by repeated contractions or by other means) may be the determining factor of the rigor changes in question—remembering that the completely fatigued excised muscle in air at 20°C. showed no recovery, and that no oxidation recovery would be possible in case of the completely fatigued intact muscles of a mammal when its circulation ceased, even if glycogen were still present in the muscles.

SUMMARY

1. Treatment of frog's sartorius muscle with Ringer's solution containing physiological amounts of sodium lactate (pH 5.2 and up to pH 8.4), for suitable periods of time, caused the treated muscle to enter heat rigor at a higher temperature and to shorten less in the first step of rigor than its mate which had been kept for the same period of treatment in ordinary Ringer's solution (pH 8.4). At pH 9.4, the rigor temperature of the muscle treated with sodium lactate in Ringer's solution was practically unchanged; but at pH 9.8 and above, such a solution caused the treated muscle to enter heat rigor at a lower temperature and to shorten less than its mate. A standard NaOH solution was used to change the pH of the acid "sodium lactate Ringer's solution."

2. Lactic acid, hydrochloric acid or sodium acid phosphate added to Ringer's solution, to give a pH about 5.4, had the same effect on sartorius muscle as described above for acid sodium lactate in Ringer's solution. Sodium bicarbonate, or sodium, potassium, calcium or ammonium hydroxides, on the other hand, when used with Ringer's solution to give a proper pH value in case of each compound (pH between 9.3 and 10.9) influenced a

treated sartorius muscle in the same manner as fatigue, i.e., caused it to enter heat rigor at a lower temperature and to shorten less than its normal mate. However, the influence of the alkaline Ringer's solution was progressive in lessening the ability to shorten, so that after a sufficient period of treatment, the muscle was rendered totally incapable of shortening during the first step of heat rigor.

3. Calcium hydroxide (pH 9.3−) in Ringer's solution was most efficient in lowering the rigor temperature.

4. Alkaline sodium hydrogen phosphate in Ringer's solution (about 2 per cent), even after the pH of the solution had fallen from 9.4 to 8.6 on standing in air, was still able to influence treated muscle in exactly the same manner as did the different modified hydroxide Ringer's solutions, except that the action of the solution was slower and the reduction of the rigor temperature was less as the pH became lower and approached 8.4.

5. Muscle which was treated with Ringer's solution containing alkaline sodium hydrogen phosphate, until after it ceased to respond to electrical stimulus, was restored by a few minutes' treatment with a Ringer's solution containing excess $CaCl_2$ (M/32) so that it responded again to electrical stimulation with definite contraction.

6. Excess $CaCl_2$ (M/4) in Ringer's solution (pH 8.4), when used to treat fatigued muscle, could reverse the effect of fatigue on rigor temperature and cause the fatigued muscle to require a higher temperature before beginning heat rigor. Also, excess $CaCl_2$ (M/16), when added to alkaline $Ca(OH)_2$ Ringer's solution in suitable concentration, prevented the usual influence of the alkaline Ringer's solution on muscle, and caused it to require even a higher temperature for heat rigor. Some evidence is given tending to show that the calcium ion was responsible for this influence of excess $CaCl_2$.

7. Various apparent contradictions are pointed out, in findings concerned with effects of different influences on muscle, in a general discussion.

8. It is shown that, of themselves, loss of glycogen and loss of lactacidogen (lactic acid precursor) from fatigued muscles (and presumably, also, from alkaline treated muscles) can not be responsible for the lowered temperature of heat rigor.

9. Evidence is presented to explain why carbonic acid in the muscle (from pure moist CO_2 gas around the muscle) differed in its effect upon heat rigor from other acids, as lactic acid for example.

10. Evidence is given for the suggestion that the lowered temperature of heat rigor, following fatigue and following treatment of the muscle with alkaline Ringer's solution of suitable pH, may be due to a higher than ordinary concentration of inorganic phosphate in the muscle (brought about by the breakdown of hexose phosphate, etc.) under conditions that tend to inhibit, or make impossible, "oxidation recovery" of the muscle.

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EFFECTS OF DIFFERENT FOOD SUBSTANCES UPON EMPTYING OF THE GALL BLADDER

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Though it is well known that the gall bladder empties in response to the ingestion of certain foods, a detailed study of the effects of a large variety of different food substances has never been made. Boyden while examining cats for anatomical variations noted the presence of collapsed vesicles while digestion was going on and was led to the discovery that feeding certain natural foods induces emptying of the gall bladder (1). A group of men working with cholecystography at the Peter Bent Brigham Hospital accidentally made the discovery that a meal rich in fat is followed by emptying of the human gall bladder and made clinical application of this finding as a sign to determine normality of the organ (2). Boyden has held that egg yolk has a specific effect upon the gall bladder of the cat (1), while we have demonstrated that complete emptying can be produced in cats by the ingestion of pure fat (3). For a thorough understanding of the physiology of the gall bladder, however, it seems necessary that an investigation of the effects of numerous food substances be made. Those studied in this paper are: Olive oil (clear and emulsified); oleic acid (clear and emulsified), cod liver oil (clear and emulsified), coconut oil (emulsified), palmitic acid (emulsified), iodized poppy-seed oil (clear), egg yolk, butter, cream; corn starch, glucose, rice (polished), macaroni; lean meat, egg-albumin, peptone (Witte's), casein, skim milk, dried and canned peas.

Different methods have been employed: that of Boyden, consisting of sacrificing cats at varying intervals after feeding and noting the state of the gall bladder and digestive organs; cholecystography in dogs; and our method of filling the gall bladder in cats with iodized oil for observation by x-ray. All these procedures have the advantage that normal animals are used. Boyden's necropsy method allows direct observation of the viscera, but only at the termination of the experiment, and slight changes can not be noted. Cholecystography allows continuous observation, and the same animal can be reëmployed, though slight emptying cannot be

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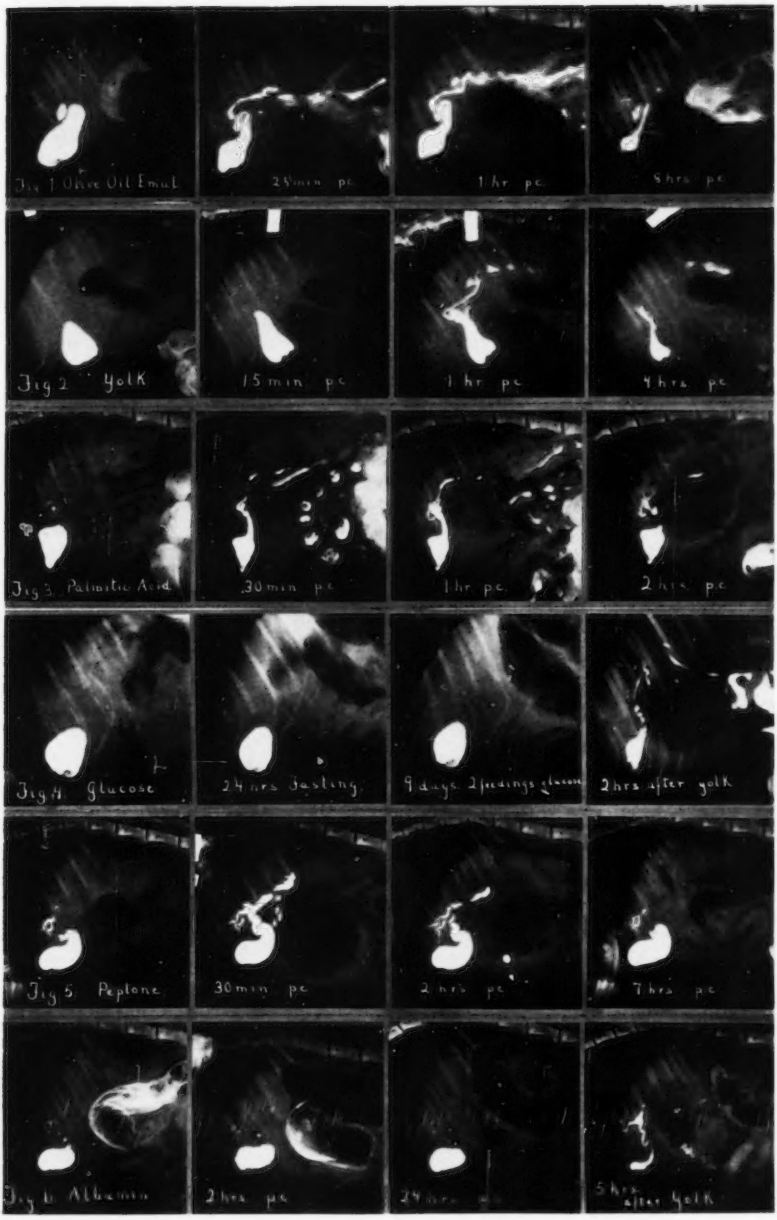

Figs. 1–6. Comparative effects of different foods upon the gall bladder of the cat

observed. The iodized oil method allows continuous observation of minute changes, but a second experiment cannot be done unless there has been no effect in the first. With a view to utilizing the advantages and minimizing the disadvantages, all these methods have been employed.

The food substances will be discussed according to classes.

Fats: Olive oil (clear), 6 cats, 15 to 100 cc., iodized oil method. In four cats the gall bladder had discharged from one-half to three-fourths of the contents after eight hours; in two there was no emptying, oil being defecated by one of them.

It is interesting to note that the clear olive oil was only moderately effective in emptying the gall bladder and that in one case it went through the alimentary tract without effect. The lacteals were pale, denoting failure of absorption of the oil.

Olive oil (emulsified), 17 cats, 40 to 100 cc., necropsy and iodized oil methods. In four cats the gall bladder had completely emptied after four to eight hours; in nine it was one-half to four-fifths empty after four to ten hours (fig. 1); in two there was slight emptying after five to eight hours, and in two others there was no definite emptying after four to seven hours.

In those cases where emptying was absent or slight, the lacteals were colorless or pale white; with pronounced emptying they were very white, indicating that the degree of absorption of fat is probably related to the degree of emptying of the gall bladder.

It is interesting to note that the animals varied greatly both in the degree of emptying of the vesicle and in the absorption of fat.

Oleic acid (clear), 7 cats, 10 to 75 cc., iodized oil method. In one cat the gall bladder was nine-tenths empty after four hours; in three it was two-thirds empty after four hours; in one there was very slight emptying, and in two no emptying in twenty-four hours, one of these being in poor condition.

Oleic acid (emulsified), 10 cats, 75 to 100 cc., iodized oil method. In five cats emptying was very prompt and the gall bladders were one-half to two-thirds empty after two hours but after that the process was generally delayed. In one cat there was no emptying after three hours but three days later the vesicle had emptied. In four cats there had been no emptying at the end of 24 hours, though there were signs of muscular activity of the gall bladder in two of them.

Cod liver oil (clear), 4 cats, 50 to 75 cc., necropsy method. In two cats the vesicle was one-fourth empty after four hours but in two others there was no emptying. The lacteals in all were pale white, denoting slight absorption of the oil.

Cod liver oil (emulsified), 15 cats, 50 to 100 cc., necropsy method. In one cat given 100 cc., there was complete emptying after four hours. In

seven cats the gall bladder was one-third to three-fourths empty after four to five hours. In two there was slight emptying after four hours. These two cats vomited the oil after one-fourth and two hours. Two other cats in which there was no emptying vomited the oil after two hours. In one of these the lacteals were colorless but in the other they were white. In four other cats in which the oil was well borne there was no emptying after four to seven hours. But in these cases the lacteals were white.

This seems an exception to the rule that absorption of fat causes emptying of the gall bladder. The rule may be that without absorption, no emptying, but there may be absorption without emptying. However, these cases were done by the necropsy method, which is not strictly accurate.

Egg yolk (with water), 10 cats, 2 to 3 yolks, iodized oil method. In one cat the vesicle discharged its contents in 15 minutes with only a slight residue in the ducts, and in another it was nine-tenths empty after 20 minutes, but here emptying stopped. In two cats it was empty after seven to ten hours. In five it was one-half to four-fifths empty after four hours, but there was no further emptying after twenty-four hours (fig. 2). In one cat there was no emptying, but this animal was in poor condition and vomited the food.

It is to be noted that there was extremely rapid emptying in some cases, slow emptying in other cases, and slow incomplete emptying in still others.

Cream (sour), 100 to 150 cc. In two cats the gall bladder was found by the necropsy method to be one-third empty after four hours, the lacteals being pale white. Of four cats with the iodized oil method, two emptied completely in 40 minutes, and in four hours. In one cat the vesicle was one-half empty after ten hours, and one which vomited the cream after two hours showed no emptying.

The same wide variation is noted with cream as with other fats.

Butter, 2 cats, 30 cc. necropsy method. In one cat the vesicle was one-half empty after four hours, in the other there was no emptying after eight hours.

Butter and sour cream, 2 cats, 50 cc., iodized oil method. In one cat the gall bladder was two-thirds empty after 15 minutes and completely empty after three hours. In another there was no emptying.

Thus the same material produced in one animal very rapid and complete emptying, and in another none.

Iodized oil (clear), 2 cats, 15 to 20 cc., iodized oil method. In one cat there was slight emptying after four hours. In the other there was no emptying. The iodized oil was seen to pass through the alimentary tract in three hours without effect upon the gall bladder. However, the vesicle had discharged two-thirds of its contents ten hours after the ingestion of egg yolk.

This illustrates again that material, even fat, can pass through the alimentary tract without effect upon the gall bladder (3).

Coconut oil (emulsion), 5 cats, 50 to 100 cc., iodized oil method. In four cats the gall bladder was one-third to one-half empty after one hour, but then the action stopped. In one cat there was slight emptying after one hour.

Palmitic acid (emulsion or suspension), 3 cats, 50 cc., iodized oil method. In one cat the vesicle was one-third empty after one hour, but there the process stopped (fig. 3). In two cats there was very slight emptying after two hours. Thus highly saturated fats and fatty acids would seem to be less effective in emptying the gall bladder than the others, though possible differences in absorption and natural variation in effects upon animals must be considered.

Carbohydrates: Corn starch, 3 cats, 100 cc. thick suspension, iodized oil method. No emptying after 24 hours. No effect upon the gall bladder.

Glucose (25 per cent solution), 7 cats, 75 to 150 cc., iodized oil method. No emptying after 24 hours. No effect upon the gall bladder. In figure 4 the first two roentgenograms show no emptying after 24 hours of fasting. The third roentgenogram shows no change after nine days, two feedings of glucose having been given at intervals, but the fourth roentgenogram shows rapid emptying after feeding egg yolk.

Cholecystograms were produced in three small dogs which were then fed 100 cc. of 25 per cent glucose. In one of these dogs there was slight reduction of the shadow after one hour upon two different occasions. In the other two dogs there was no reduction of the shadow after one hour upon two different occasions, though reduction took place promptly after feeding egg yolk.

Thus in only one of thirteen animals did carbohydrates produce any effect upon the gall bladder. The exception prevents us from making the rule that carbohydrates produce no emptying of the gall bladder, but the effect in any case if present is very slight. This is of importance clinically in view of the fact that glucose feeding has been recommended as a test for normality of the gall bladder in cholecystography (4). It is obviously of no value in this respect.

Macaroni (boiled), 3 cats, 100 cc. Thick suspension, iodized oil method. No emptying after 24 hours. No effect upon the gall bladder.

Rice (polished, boiled), 3 cats, 100 cc. thick suspension, iodized oil method. In two cats the gall bladder was one-half empty after one hour and in the other, three-fourths empty after two hours.

Thus a mixed carbohydrate food like rice is moderately effective in emptying the gall bladder.

Proteins: Lean meat, 3 cats, iodized oil method. The gall bladder in one was approximately three-fourths empty after 20 hours; in another

three-fourths empty after twelve hours and in the third one-half empty after 24 hours.

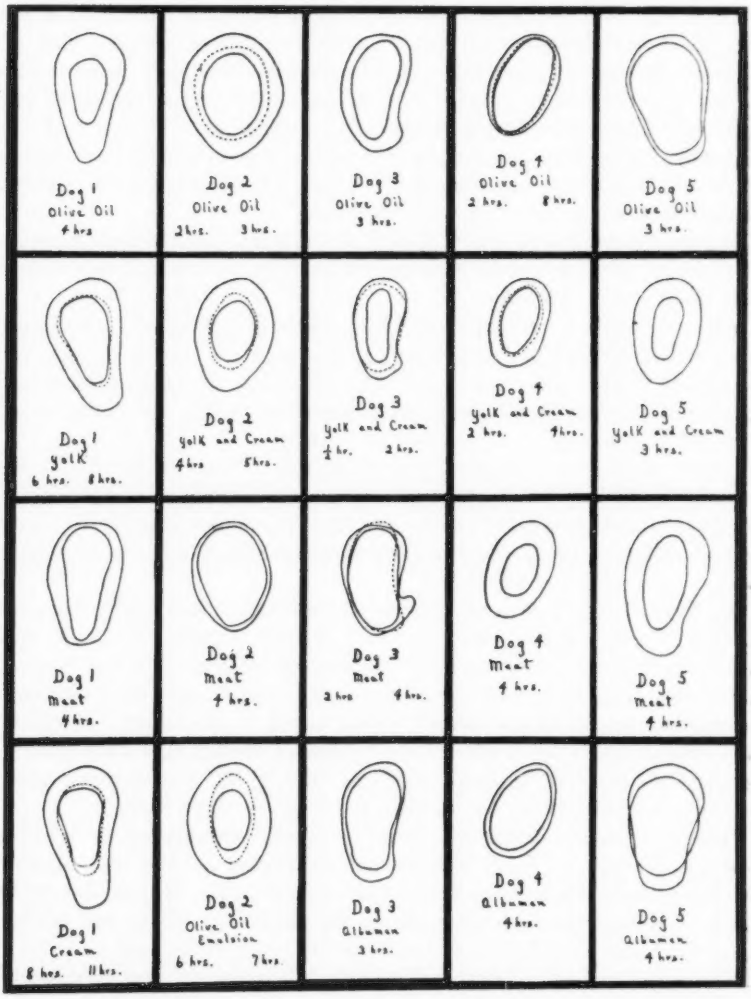

Fig. 7. Comparative effects of different foods upon the gall bladder of the dog

Peas (both canned and dried), 6 cats, 80 to 100 cc. thick suspension, iodized oil method. In four cats the gall bladder was from one-third

to three-fourths empty after four hours; in one there had been very slight emptying and in another no emptying at the end of 24 hours.

Peptone (Witte's), 8 cats, 50 to 100 cc. in thick suspension, iodized oil method. In two cats there was very slight emptying after two hours (fig. 5); in one there was no emptying after six hours, though olive oil emulsion subsequently provoked it. In the other five cats, there was no emptying after two to seven hours, though subsequent feeding of egg yolk was followed by varying degrees of emptying.

Casein, 2 cats, 50 cc. thick suspension, iodized oil method. In both cats there was evidence of contraction of the gall bladder, consisting of filling of the cystic duct, but no definite emptying.

Skim milk (centrifugalized), 6 cats, 40 to 200 cc., iodized oil method. In two cats the gall bladder was one-third empty after two hours; in four cats there was no emptying.

Albumin (egg), 6 cats, 100 to 150 cc., iodized oil method. Three cats were fed the albumin partly cooked and whipped up. In one of them the gall bladder was one-fourth empty after two hours; in another there was an attempt at emptying, indicated by elongation; and in the third, which vomited after one hour, there was no emptying.

Three cats were fed the albumin raw, shaken up with a little water. In one of them there was very slight emptying after one-half hour, but nothing further. In the two others there was no sign of emptying for 24 hours, though one subsequently emptied after egg yolk (fig. 6) and one after olive oil.

In figure 7, the comparative effects of different foods upon the same animal at different times can be seen. In each figure the larger solid outline represents a tracing of the cholecystogram in a dog before feeding; the dotted line, an intermediate tracing; and the smaller solid outline a tracing at the end of the longest period noted on the figure. It will be observed that fats are in general most effective, though they vary greatly; meat is less effective, though in one case (dog 4) its effect is pronounced; albumin produces slight but definite reduction in three cases.

The question has arisen as to whether psychic stimulation, such as the sight and smell of food, will produce emptying of the gall bladder. McMaster and Elman have stated that it occurs (5). If true this would be a very important consideration in the physiology of the gall bladder in general, and would greatly complicate its reactions to food.

But since the gall bladder will remain full for days, and even weeks, during fasting (6) it would seem unlikely that psychic stimuli produce emptying. However, six cats with the gall bladder full of iodized oil were fasted until very hungry and then exposed to the sight and smell of canned salmon for one hour. All evinced great interest in the food and some were very active in their efforts to reach it. Some of them were even allowed

to lick the fingers that had handled the food. In no instance was there the slightest emptying of the gall bladder.

SUMMARY

1. Fats and fatty acids are by far the most active foods in emptying the gall bladder and the unsaturated appear more effective than the saturated.

2. Pure carbohydrates are practically ineffective in emptying the gall bladder. In only one of thirteen animals was slight emptying noted.

3. Pure proteins produce slight emptying of the gall bladder.

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THE INFLUENCE OF LOCAL DIATHERMY ON GASTRIC MOTILITY AND GASTRIC SECRETION

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The literature on the action of diathermy on the motor activity of the stomach is sparse. Working on dogs, Stewart and Boldyreff report that the application of diathermy during the periodical contractions of the empty stomach (hunger contractions) produces ordinarily cessation or weakening of these contractions. Considerable work on the action of high frequency currents on gastric secretion has been reported in the last years. Most of this is clinical and the results conflicting.

Ludin studied the effect of diathermy on gastric secretion in man. He previously had verified the increase of local temperature in the stomach upon the application of diathermy, this increase reaching a maximum of 2.1°C. His experiments, however, indicated that diathermy did not influence the quality or quantity of gastric secretion. On the other hand, Stewart and Boldyreff, working with Heidenhain pouch dogs, concluded that diathermy increases the acidity of both continuous and digestive secretion but apparently diminishes the pepsin content. They also state the diathermy increases the gastric secretion in the "psychic stage" as well as in the "chemical stage" of digestion. Brody, treating dogs with diathermy under morphine-ether anesthesia, reported that the immediate effect of the diathermy was an increase in both the free and the total acidity. This increase was followed by a period of depression. It may be significant to note that the active electrode employed by Brody consisted of a metallic bulb passed directly into the stomach *per os*. Setzu studied several cases (man) of gastric disorders under diathermy therapy. He concluded that diathermy does not increase markedly the gastric acidity.

These conflicting results are representative of the current literature.

METHODS. The balloon method was used for recording the hunger contractions in gastric fistula dogs. In order to establish constant conditions the dogs were maintained on constant diet and fed at the same hour each day. Tracings were recorded at about 16 hours after a meal, during a period of 3 to 5 hours. The applications of diathermy were made either during the period of hunger contractions or during the period of

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quiescence. The high frequency current was produced by the machine manufactured by the Victor X-ray Company, commonly used for therapeutic purposes in medical practice. Lead electrodes of 6 × 8 for one dog, and 5 × 6 cm. for the others were placed on lateral aspects of the abdomen so as to include the stomach between the electrodes. Dogs were shaved locally and good contact was assured by soaping the skin and by adapting electrodes with sand bags or held taut by drawn gum tubing. Generally, intensity 2, voltage C, frequency 4 and from 2 to 3 spark gaps were employed in order to furnish milliamperages varying from 500 to 1000 milliamperes. The current was applied for about 30 minutes. It has been found that these doses were well borne, the dogs resting comfortably and suffering no burns. With higher doses the dogs became restless.

Pavlov pouch dogs were used in gastric secretion tests. The technique of application of diathermy was the same as in the gastric motility tests.

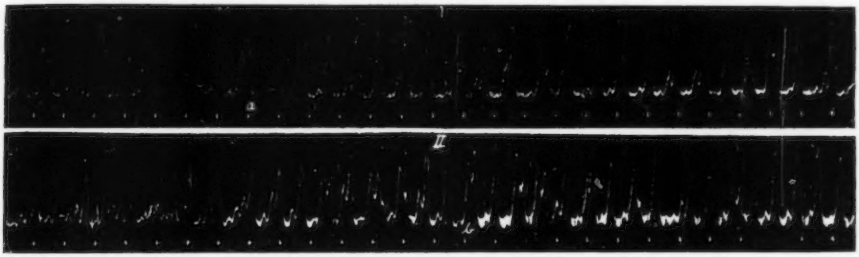

Fig. 1. Dog. Water manometer tracings of the contractions of the empty stomach. Reading from left to right, II is a direct continuation of I. *a*, beginning diathermy (750 m.a.) of the stomach; *b*, end of diathermy. Showing no influence of the diathermy on the tonus and the hunger contractions. Time: minutes.

With such technique the effect of 30 minutes' application of diathermy was determined on the fasting secretion and on the secretion after a standard meat meal (100 grams ground lean meat). Control observations were made on the day preceding and on the day following the diathermy treatment under identical conditions. Samples of gastric juice were collected at half-hour intervals for a period of 2½ hours. Gastric juice was obtained in the first series of experiments by drainage and in the second series by continuous aspiration. The last method has proven the most satisfactory. Besides being more accurate there is practically no leakage of gastric juice; it permits the collection of gastric juice during the application of diathermy with the dog in lateral decubitus.

The acidity was titrated against N/40 NaOH solution. Dimethyl amino-azo-benzene and phenolphthalein were used as indicators of free and total acidity respectively. The results are expressed in clinical units.

THE INFLUENCE OF DIATHERMY ON MOTILITY. Dog I. Young, 9 kgm. About one week after the gastrostomy operation controls were run for six days. The hunger contractions were of type I or type II, but more constantly of type II. The quiescent periods lasted about one-half an hour. Hunger contraction periods of 1½ to 2 hours were regularly shown by this dog.

Seventeen applications of diathermy currents (milliamperage from 500 to 950 milliamperes) were made during hunger contraction period for 20 to 30 minutes. Respiration rate increased. No modification in strength or rate of hunger contractions or in the tonus of stomach was obtained. Six applications were made during quiescent periods. No modification in length of quiescent period or in tonus was observed.

Dog II. Young, 10 kgm. Hunger contractions of type I or II, more frequently type II, were typical for this dog. Five controls were run before any application of diathermy.

Ten applications of diathermy currents were made with milliamperage varying from 600 to 950, for 15 to 40 minutes, generally 30 minutes, during hunger contraction period. There was no modification of hunger contractions. Four applications were made during quiescent periods with equally negative results.

Dog III. Adult, 14 kgm. Sixteen controls were run before diathermy. Hunger periods lasted about one-half hour, quiescence period about 1½ hours. Six applications of diathermy from 400 to 1000 milliamperes were made during hunger contraction periods. No modification of hunger contractions was observed.

DISCUSSION. Two conditions have always been taken into consideration; maintenance of the dog in a comfortable resting position on the one hand, and, on the other hand, the administration of sufficient current to produce an effect. The first condition can be more easily assured. Dogs are quickly trained to tolerate the application, eliminating the excess heat by panting, but staying perfectly quiet.

The second is more difficult to establish. It is well known that the density of the electrical current passing through a given organ is practically impossible to measure. In practice the quantity of current passing through an organ is usually measured by the heat produced. Concerning diathermy on the stomach, much work has been done on man, the temperature of the stomach being measured by thermometer or thermo-couples (for literature, see Ludin).

We measured the heat produced by the diathermy in the stomach of our dogs by means of sensitive thermometers introduced into the stomach through the fistula. Rectal temperature was simultaneously recorded. The temperature in the stomach reached a maximum increase of 0.6°C. (current of 500 to 950 milliamperes). At that level the dog starts to pant,

thus eliminating the excess heat. In our experience it is not possible in dogs not under an anesthetic to raise the intragastric temperature more than 0.6°C. The rectal temperature was either unchanged, or increased by about 0.2°C.

The increase in the stomach temperature by diathermy is, thus, very small. We found that a temperature increase in the stomach, as high as the maximum produced by diathermy, could be produced by allowing the dog to play for a short time.

TABLE 1

Pavlov pouch dogs, summary of experiments on the influence of diathermy on gastric secretion

GASTRIC CONDITION	GASTRIC SECRETION CONTROLS				GASTRIC SECRETION DURING AND AFTER DIATHERMY			
	Number of experiments	Volume	Free HCl	Total HCl	Number of experiments	Volume	Free HCl	Total HCl
		cc.				cc.		
Dog I:								
Fasting 30 minutes	33	2.3	27	57	20	2.0	25	56
100 grams meat:								
30 minutes	33	3.8	67	83	20	4.6	52	93
30 minutes	33	4.2	85	106	20	4.0	88	103
30 minutes	33	3.7	87	111	20	4.0	74	105
30 minutes	33	3.0	82	109	20	3.5	76	102
Dog II:								
Fasting 30 minutes	24	1.0	15	55	22	1.0	7	25
100 grams meat:								
30 minutes	24	2.0	44	68	22	2.0	37	58
30 minutes	24	3.1	77	60	22	2.7	85	110
30 minutes	24	2.6	86	111	22	2.6	85	120
30 minutes	24	2.3	97	116	22	2.2	87	129

With the dog under paraldehyde anesthesia—consequently under unfavorable conditions to eliminate heat—the increase in the local temperature of the stomach reached 1°C. with application of 400 milliamperes. It seems that in normal conditions, as soon as the temperature rises, the dog starts to pant, and by means of this mechanism prevents the temperature from rising further. The diathermy temperature rise is not sufficient to induce the inhibition of gastric motility seen in fever.

High frequency currents applied in amounts equivalent to those used in clinical practice and sufficient to produce polypnea (provided that the exposure does not cause discomfort to the animal) have no effect on the

motor activity of the empty stomach. A slight depression of the contractions may appear just before or at the beginning of panting.

THE INFLUENCE OF DIATHERMY ON GASTRIC SECRETION. The results are summarized in table 1. We believe that number of control and diathermy tests make the average figures significant. No distinct influence of the diathermy on the quantity and acidity of gastric secretion is shown by our data. This applies to acute or immediate effects. The individual protocols similarly fail to reveal any chronic effects on gastric secretion in consequence of repeated local application of diathermy.

CONCLUSION

In dogs not under anesthesia diathermy applied to the shaved abdominal wall of an intensity and duration so that the temperature of the interior of the stomach is raised to the maximum of 0.6°C. has no influence on the gastric secretion (fasting, digestion) or on the gastric tone and contractions (empty stomach). We know that, within limits, an elevation of the temperature augments some of the processes in isolated organs or tissues. In the normal intact animal this influence of the slight rise in gastric temperature possible to induce and maintain by diathermy is evidently checked by other factors.

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MUSCLE HEMOGLOBIN CONCENTRATION DURING GROWTH AS INFLUENCED BY DIET FACTORS

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When it became apparent that in dogs we could control the regeneration of blood hemoglobin by diet factors we determined to attempt a similar diet control of the construction of muscle hemoglobin during growth periods when presumably the maximum production of muscle hemoglobin was to be observed. It is so easy to demonstrate diet control of blood hemoglobin regeneration in standard anemias that we blithely took up this muscle hemoglobin problem with eager anticipation of easy diet control of muscle hemoglobin levels in growing dogs. We began this work three years ago and feel that it can be said without fear of contradiction that the control of muscle hemoglobin concentration in the growing pup is far from simple.

The experimental data cited below indicate a difference between the pups raised on a liver diet and those raised on a bread mixture diet, the former showing somewhat higher muscle hemoglobin concentration. These experiments indicate again that the circulating blood hemoglobin is a *labile substance* subject to fluctuations, rapidly rebuilt and readily controlled by diet factors but by contrast muscle hemoglobin is a *stable substance* not subject to wide fluctuation even in conditions of anemia or of wide divergence of diet intake. We have pointed out (2) that blood and muscle hemoglobins are almost indistinguishable chemically but have distinct individualities when tested by specific precipitin tests (1). The experiments tabulated below bring out other physiological differences between blood hemoglobin and muscle hemoglobin.

Our experimental plan was to take litters of pups just after weaning and place half the litter on a bread diet, unfavorable for blood hemoglobin regeneration and the rest of the pups on a diet highly favorable to blood hemoglobin regeneration (heavy liver feeding). We expected to find in a few weeks that the muscle hemoglobin in the two groups would show large differences in concentration in the skeletal muscle. We have a considerable number of observations to show that diet periods of 10 weeks will give no uniform differences in the content of muscle hemoglobin in these two groups. Pups which have been on these diets for 25 to 30 weeks

will show distinct differences—sometimes as much as 45 per cent greater muscle hemoglobin volume in the liver-fed animal.

METHOD. These litters of pups were born in our own kennels and always nursed about six weeks. At all times they were kept with the mother in a warm well-ventilated and lighted room, bedded with shavings, with plenty of room for exercise. After weaning the mother is taken away and the pups put on a bread and milk diet for a few days. Some time in the seventh week the pups are separated into two groups and placed upon a fixed ration. One group receives liberal amounts of a salmon-bread and a whole milk powder (Klim) mixed into a mash with warm water. The other group receives half the bread ration of the first group and half of cooked ground pig liver mixed into the same mash.

According to our experience in anemic dogs the salmon-bread, milk mixture is unfavorable to hemoglobin production but adequate for general maintenance. The liver diet is very favorable to blood hemoglobin regeneration in anemia. The *salmon-bread* is prepared and baked in our laboratory and has been described elsewhere (5). It consists of wheat flour, starch, bran, sugar, cod liver oil, canned tomatoes, canned salmon, yeast and salt mixture. The pups thrive on these diets and show uniform growth curves. One lot raised in mid winter had a little trouble with rickets which was readily cured by carbon arc light therapy and cod liver oil. The pups are weighed each week and a sample of blood obtained from the jugular. The red cell hematocrit and hemoglobin values are determined from such samples as described elsewhere (5).

One of the great difficulties in this work is the constant menace of distemper infection. The pups are kept in stalls in a separate room upstairs while all adult dogs are kept downstairs. They are cared for by the upstairs janitor but in spite of all precautions many litters become infected with distemper which is usually promptly fatal to young pups. Muscle hemoglobin determinations done after distemper developed were unsatisfactory and we observed blood concentration, tissue dehydration associated with the bronchopneumonia and enteritis usually found in these infected pups. These values (distemper) therefore have no significance as to the normal blood hemoglobin values or normal muscle hemoglobin concentration and are not included in this paper. All the pups included in table 1 were quite normal when killed by ether anesthesia. The age column gives the total age in weeks since birth so that the period on the stated diet is *seven weeks less* than the stated age. The hemoglobin values are read against a standard with 100 per cent equivalent to 13.8 grams hemoglobin per 100 cc.

The extraction of muscle hemoglobin in quantitative amounts has been described (4). In brief the anesthetized pup is bled slowly from the carotid while a simultaneous equivalent infusion into the jugular vein of

a 5 per cent sugar Locke's solution is given. By this method the red cell hematocrit is usually reduced from 40± per cent or normal to about 1 per cent or less. When the heart ceases to beat the thorax and abdomen are opened at once and all viscera removed en masse. The legs and back are then massaged to drive out as much residuum of diluted blood perfusate as possible. Samples are then dissected from the various muscles as expeditiously as possible. These samples are rinsed and massaged in normal saline, dried with a towel, weighed and cut up finely to be extracted in 0.4 per cent ammonia and distilled water. The extraction is carried out in an ice box for 15 to 20 hours after which time the red solution is measured either as carbon monoxide hemoglobin or as acid hematin.

TABLE 1

Muscle hemoglobin and diet in pups—mgm. per 100 grams muscle

DOG	AGE	WEIGHT	DIET		BLOOD HEMOGLOBIN	MUSCLE HEMOGLOBIN, AVERAGE
			Bread	Liver		
	weeks	*kgm.*			*per cent*	
25-104	23	7.7	+	0	85	371
25-102	22	8.3	+	+	95	411
27-16	25	5.5	+	0	58	237
27-12	25	6.0	+	+	85	282
27-17	26	6.9	+	0	58	263
27-13	26	6.9	+	+	72	309
27-15	34	13.2	+	0	78	460
27-14	34	9.2	+	+	96	529
25-96	19	8.1	+	0	70	337
25-99	19	8.0	+	+	77	320
25-95	37	15.9	+	0	88	479
25-98	35	17.3	+	+	100	706

Acid hematin values are given in these tables. The values given in table 1 are averages of several muscles all analyzed separately as described. In these *averages* we include muscle samples from triceps, flexors, adductors, gastrocnemii and spinae of both right and left sides.

EXPERIMENTAL OBSERVATIONS. The weight of the pups at the start of these experiments varies a great deal with the breed of pup or rather the breed mixture but averages 1.5 to 2.5 kilos. The gain in weight in normal pups on these two diets is steady and uniform unless there is some complication—intestinal worms, for example. The level of blood hemoglobin at the start of these experiments is almost exactly 50 per cent with fluctuations of 5 per cent each way. The gain in blood hemoglobin shows a slowly mounting curve with some irregularities which cannot be explained.

The liver fed pups make distinct gains in blood hemoglobin level over the bread fed pups. So far as we can see both groups are clinically normal and active and mature at the same time as do groups on the usual mixed diet.

A perusal of table 1 makes it evident that diet periods of 20 to 25 weeks will show a distinct though usually moderate difference in favor of the liver fed pups both in blood hemoglobin and muscle hemoglobin concentration. The figures on which the average muscle hemoglobin values of table 1 are based are illustrated by 4 type experiments given in table 2.

The figures in table 2 supplement the values given in an earlier publication (4) to establish the normal base line of muscle hemoglobin concentration

TABLE 2

Muscle hemoglobin and diet in pups—mgm. per 100 grams muscle

	DOG 27-14	DOG 27-15	DOG 25-98	DOG 25-95
R. rectus	410	385	547	376
L. rectus	406	374	677	363
Diaphragm	511	431	710	532
Heart	321	310	360	374
R. triceps	476	390	726	472
L. triceps	495	396	710	460
R. flexor	585	513	790	535
L. flexor	561	505	805	550
R. gastrocnemius	539	495	730	460
L. gastrocnemius	565	482	750	465
R. adductor	539	482	640	450
L. adductor	552	460	620	475
R. spinae	486	430	660	463
L. spinae	493	448	630	460
Average	529	460	706	479
Diet	Liver	Bread	Liver	Bread
Blood hemoglobin, per cent	96	78	100	88
Age, weeks	34	34	35	37

at various ages in pups. The reasons for the differences observed in heart, diaphragm, flat muscles and leg muscles have likewise been discussed and need not be repeated.

The first two pups in table 2 deserve special consideration for several reasons (dogs 27-14 and 27-15). This litter was a mongrel litter which showed more type divergence than usual but the liver-fed pup chosen in each instance was the smaller. Moreover, this litter suffered a little from rickets as it was raised during mid-winter. The liver-fed pups showed more evidence of clinical rickets than did the bread controls but carbon arc therapy and a little excess of cod liver oil cured the rickets within one to two months. The oldest liver-fed pup was much below its bread con-

trol mate, 9.2 kgm. liver and 13.2 kgm. bread. We felt that the larger bread fed pup was a little more active and certainly more powerful but in spite of this unfavorable difference the muscle of the liver-fed, smaller, less active dog contained more muscle hemoglobin than did the bread control.

As illustrative types we are recording the autopsy findings of these two pups which are typical and of especial interest in the liver fed pup.

Autopsy 27-15, standard salmon-bread diet. Very healthy, active, vigorous, well nourished mongrel, just coming to sexual maturity. This dog is more active and powerful than its mate which was fed liver so that it would be probable on the score of physiological and muscular activity that this dog would exceed the other in spite of the handicap of the bread diet. Perfusion as usual. Injection 2250 cc. Perfusion carried to about the same extent as in the other dog. Viscera showed no pigmentation whatever. Perfusion of intestines good. Mucous membranes pale. The muscle coats of the intestinal tract pale white. Lungs snow white. About the same amount of perfusion edema in this case as in the litter mate. The edema shows most about the pancreas.

Bones: All parts of the long bones show cellular marrow, there being little evidence of any fat in the middle third of the femora and humeri. The bone marrow in the tibiae and ulnae appear to be uniformly pinkish red, containing but little fat. Line of ossification at the costo-chondral junction of rib sharp and normal. This dog shows the cellular marrow involving an area apparently larger than its mate, the liver fed dog.

Dog 27-14. Autopsy. Standard bread and heavy liver feeding. Healthy active, white mongrel approaching sexual maturity. Perfusion as usual, injection 1550 cc., bleeding 1500 cc. The extent of perfusion was satisfactory and the viscera free from blood. The viscera were normal except for an *unusual pigmentation.* This color may be described as yellowish brown or "maple sugar color." In our animals it is a constant finding and in a measure deepens in intensity with the duration and amount of liver feeding. This brown coloration is most conspicuous in the pancreas and muscle coats of the small intestine where the color is almost russet. The large intestine is much less involved. The liver and kidneys in this dog were likewise moderately involved. This brown pigment is readily soluble in alcohol but calls for much more study to ascertain the significance of this pigment deposit. It may be mentioned in passing that some of these pups raised on liver have an unusual *reserve capacity* for the production of new blood hemoglobin in long severe anemia. Further work is in progress to study this significant departure from normal.

Bones: The bones are much like the control dog. No signs of rickets but a little deformity remains in the fore legs. There is, however, a distinct difference in the marrow of the leg bones where there is more fat tissue and less red marrow.

Microscopical sections from various specimens of cellular marrow in femora, humeri, tibiae, ulnae show distinct differences which may be summed up somewhat as follows—the marrow of the liver fed dog is not as cellular and contains much coarsely granular yellow brown iron staining pigment contained in large phagocytes. The marrow of the bread fed control dog is much more cellular and contains no pigment. It appears in all respects normal but somewhat hyperplastic.

We may be permitted to speculate as to the significance of these observations in marrow sections. It is possible that the pup on the bread diet unfavorable to blood hemoglobin regeneration was forced to expand his marrow boundaries to keep up the needed supply of red cells used for maintenance. The same reaction is noted when there is a moderate continued blood loss. It is possible that the liver fed pup had a considerable excess of pigment building material or pigment construction factors and was forced to store some in various body tissues as well as in the common phagocytes. These various pigments may have some potential relationship to body hemoglobin.

Discussion. One may inquire as to the amount of muscle hemoglobin which may be required during growth periods and the following values may be submitted. Many of these bull pups (table 1—25-95 and 25-98) gain steadily 0.5 kgm. per week. Of these 500 grams we may assume about 30 per cent is muscle tissue during the 4th to 8th month of life in active pups—that is, a gain of 150 grams of muscle tissue per week. If we take 500 mgm. of muscle hemoglobin as the average values for the concentration of hemoglobin in pups' muscle of this age we come to a total of 0.75 gram of muscle hemoglobin needed each week for muscle growth. We have no knowledge of the replacement values for muscle hemoglobin but we must assume a considerable wear and tear and utilization of this hemoglobin.

The striated muscle is more or less of a permanent fabric of fibrils, sheaths, nuclei and various other elements but the body must substitute various essential elements to replace worn out material without disturbing the essential fabric. The amount of new muscle hemoglobin required for growth is not large nor would it require much food material when we compare potential muscle hemoglobin and blood hemoglobin. For example in pups of this age made anemic and supplied with the liver diet we could confidently anticipate an output of *30 to 40 grams of blood hemoglobin* each week—about 50 times the requirements for new muscle hemoglobin in growth periods as outlined above.

One may debate whether the higher levels in muscle hemoglobin in liver fed pups may be due directly to food intake or to some other related factor. For example, the *blood hemoglobin level* is higher in these liver fed pups and it may be argued that the muscle hemoglobin levels depend upon this blood hemoglobin level. However, we have shown elsewhere (3) that

severe anemia must continue many weeks to influence even slightly the level of muscle hemoglobin in a normal dog and the conclusion seemed obvious that the muscle hemoglobin levels are stable and not subject to fluctuation dependent upon varying blood hemoglobin levels. Again one may question *bodily activity* for it is readily shown that extra work will call out an extra concentration of muscle hemoglobin within the affected muscle. It is not easy to control this factor and a great variety of recording apparatus would be required to give even a partial objective record of bodily activity. So far as observation goes we cannot record that the liver fed group is any more active than the bread diet group. There are individual variations in activity. It must be admitted nevertheless that the different blood hemoglobin levels as well as difference of bodily activity may be factors, as well as the direct diet intake, which bring about the difference in muscle hemoglobin levels in these two diet groups.

CONCLUSIONS

Litter mates were divided in two groups and after weaning were fed an adequate synthetic bread ration or a bread ration plus large amounts of cooked liver.

No differences in muscle hemoglobin concentration in the two groups were noted after 10 weeks' feeding.

After 15 to 20 weeks on these diets the liver fed pups showed uniformly a distinct increase in blood hemoglobin levels and muscle hemoglobin concentration above the bread controls.

After 25 to 30 weeks these differences in favor of the liver feeding may be more distinct.

Blood hemoglobin levels are labile and easily disturbed and the body capacity for producing large amounts of hemoglobin is fortunately very great.

By contrast the muscle hemoglobin levels are stable and not easily disturbed. Whether the body can produce large amounts of muscle hemoglobin must be left an open question but the growth requirements of new muscle hemoglobin are not large. The maintenance factor required to replace wear and tear of muscle hemoglobin is unknown.

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THE HEMOGLOBIN OF SMOOTH AND STRIATED MUSCLE OF THE FOWL

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During the past few years we have studied muscle hemoglobin from a variety of sources and have found absolute uniformity when comparing the muscle hemoglobin of the dog (3) and man (6). This paper gives information about the striated muscle (leg) and the smooth muscle (gizzard) of the domestic fowl. It is readily seen that the spectrophotometric analysis of the muscle hemoglobin of the fowl is identical with that of the dog. This means that these substances are very much alike if not identical, at least in the pigment portion of the large hemoglobin molecule. Probably delicate precipitin tests would readily differentiate between these hemoglobins of the dog and fowl as can be done between blood and muscle hemoglobins of the dog (1).

Smooth muscle hemoglobin is not a common finding in animals. We are familiar enough with the fact that striped muscle may contain more or less of an intrinsic pigment hemoglobin but the presence of hemoglobin in smooth muscle is unusual. Most of the smooth muscle of the common laboratory animals is free of hemoglobin. The gizzards of birds are notable exceptions and this smooth muscle usually contains much more muscle hemoglobin per gram than do the skeletal striped muscles.

Gizzard muscle is peculiar then in that it contains a high concentration of muscle hemoglobin whereas the rest of the smooth muscle of the birds' intestinal tract is innocent of any appreciable amount. It is possible that the large amount of heavy work done by the gizzard has been responsible for this store of muscle hemoglobin. We (4) have shown that the concentration of muscle hemoglobin in striped muscle is largely determined by muscle activity and the highest content is found in the leg muscle of the highly trained and conditioned hunting dog. Another interesting characteristic of the gizzard may be mentioned as pertinent to this thesis. The gizzard as a food is much more potent than skeletal muscle in its capacity to produce new blood hemoglobin in standard anemic dogs (5). For example, the feeding of beef muscle to a standard anemic dog may average a production of 20 to 25 grams hemoglobin per two week period over and

above control periods. Under identical conditions the feeding of cooked gizzard in equivalent amounts for two weeks may show 70 to 85 grams hemoglobin production above control periods. This indicates the presence of substances in this gizzard smooth muscle which are readily built into blood hemoglobin. It may be that these materials are reserve storage of pre-pigment material available for replacement of the muscle hemoglobin of the gizzard which does such heavy work with probable rapid wear and tear of its hemoglobin.

The experimental methods have been described fully in the first paper of this series (4) and needed only minor modifications in these experiments. The spectrophotometer has likewise been adequately described (2, 3).

The animals used were large adult roosters of "Rhode Island Red" stock. Under ether anesthesia the jugular vein and a large artery at the root of the neck were dissected free. A large hypodermic needle was inserted into the jugular and the artery was cut. Gravity perfusion through the jugular of a Locke's solution plus 5 per cent glucose was continued as long as the bleeding from the carotid was observed. This removes the bulk of circulating hemoglobin in the rooster as in the dog where the red cell hematocrit may be reduced from 50 per cent (normal) to 1 per cent. The comb will be noted as well bleached in spite of the section of a large neck artery. Now the thorax and abdomen are widely opened by raising the breast bone and section of ribs. The heart is usually beating feebly. A large cannula is inserted into the left ventricle and rapid gravity perfusion is continued with mechanical pulse-like compression of the ventricle. The right auricle is incised to permit free escape of the perfusate and several liters of perfusate are washed through the circulation. An assistant massages the legs meanwhile to facilitate washing out the last traces of blood hemoglobin. The viscera will be found quite clear of blood color except the spleen. By this method one can prepare dogs' muscle hemoglobin which is free from blood hemoglobin as demonstrated by delicate precipitin tests (1). It is likewise probable that the muscle hemoglobin prepared in these fowl experiments is likewise free from blood hemoglobin contamination.

Six experiments were done in this way and the muscle hemoglobin extracts prepared as described previously (4). The minced muscle was extracted with 4 volumes of 0.4 per cent ammonia solution in an ice box over night. The red solution was filtered through cloth, centrifugalized to remove sediment and fat and filtered through paper. The clear solution was then examined by the spectrophotometer.

Except for the differences due to variable concentration of extracted pigment in the different cases the spectrophotometric curves were identical. The plots in chart A were derived by calculating the absorption ratio for each individual curve, averaging these values and plotting a composite curve which would represent an average type curve.

Curve I, figure 1, is chicken blood diluted approximately 1 to 100. If compared with blood curves derived from other species (2) no differences are to be noted. Curves II and III are derived from striped muscle (leg) and smooth muscle (gizzard) respectively. Five perfused gizzard samples were analyzed quantitatively with values of 816, 780, 624, 654 and 635 mgm. per 100 grams gizzard or an average of 702 mgm. per 100 grams. Three leg samples were analyzed quantitatively with values of

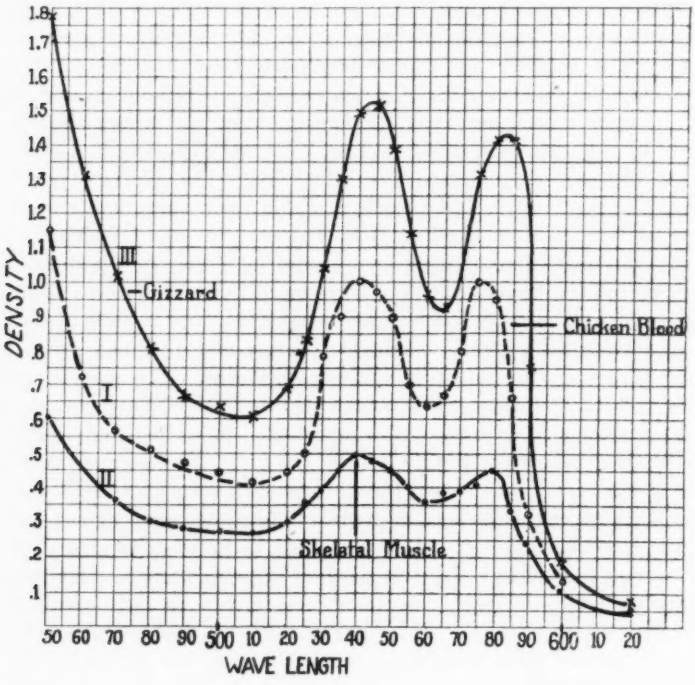

Fig. 1

342, 318 and 317. They show that there is about twice as much pigment in the smooth muscle as in the skeletal leg muscles. In addition, there is a slight shift toward the red in the bands of the muscle extract when compared with whole blood. Also there is a suggestion that the band in the yellow is slightly paler in the muscle hemoglobin extract.

These slight differences between blood hemoglobin and muscle hemoglobin have been discussed elsewhere (3). The method of extraction or slight ferment action may be in part concerned with these differences. We concluded at that time that these hemoglobins were chemically either

identical or almost indistinguishable. In view of more recent experiments with delicate precipitin tests which enable us to differentiate sharply between dog muscle hemoglobin and blood hemoglobin we may conclude that there may be differences in chemical structure. Under these circumstances these slight differences between the spectrophotometric curves of muscle hemoglobin and blood hemoglobin may have real significance and indicate a slight but real chemical difference between these important hemoglobins. We hope to report other physiological differences between these two hemoglobins.

CONCLUSIONS

Spectrophotometric analysis of chicken blood hemoglobin shows a curve identical with that of dog blood hemoglobin.

Likewise the chicken striated muscle hemoglobin analysis is identical with that of the dog striated muscle hemoglobin.

Smooth muscle (gizzard) hemoglobin appears to show the same slight differences as does dog muscle hemoglobin when carefully compared with blood hemoglobin by means of the spectrophotometer.

Smooth muscle hemoglobin is a rarity and deserves much more study. Its significance is discussed.

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REFLEXES FROM THE GASTRO-INTESTINAL TRACT TO THE LABYRINTH

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It has been shown (1, 2, 3) in dog and man that the central nervous system is affected reflexly by stimulation of the abdominal viscera. It was shown (4) in man that vision is markedly affected by gastro-intestinal distention. Patients with gastro-intestinal "upsets" are frequently abnormally disturbed by noises. We were, thus, curious to ascertain the effects that gastro-intestinal distention would produce upon auditory acuity and vestibular activity.

METHODS. The gastro-intestinal tract was distended by gently forcing air into the stomach through a Rehfuss tube. The air was slowly administered over a period of half an hour. The sensations were similar to those produced by eating a moderately large meal (2, 4), such as muscular and psychic relaxation, etc. In one experiment a large quantity of hot water was taken orally but the reflex effects upon the eighth nerve activities were exactly the same as when air at room temperature was used. In other experiments distention of the stomach with a large meal produced similar results.

The auditory acuity was determined with a Western Electric Model 1A Audiometer. The usual vestibular tests were used.

RESULTS. In every case cochlear and vestibular activities were increased. Results from typical experiments follow:

A. *Vestibular.* April 30, 1927.

Normal.

No spontaneous nystagmus or past pointing.

Rotation to right 10 times in 20 seconds. Nystagmus moderate, duration 20 seconds

Rotation to left 10 times in 20 seconds. Nystagmus moderate, duration 20 seconds

Rotation to right 10 times in 10 seconds. Past-pointing right hand 4 inches to right, left hand 4 inches to right

Rotation to left 10 times in 10 seconds. Past-pointing right hand 4 inches to left, left hand 3 inches to left

One-half hour after beginning distention of the stomach.

Rotation to right 10 times in 20 seconds. Nystagmus violent, duration 26 seconds

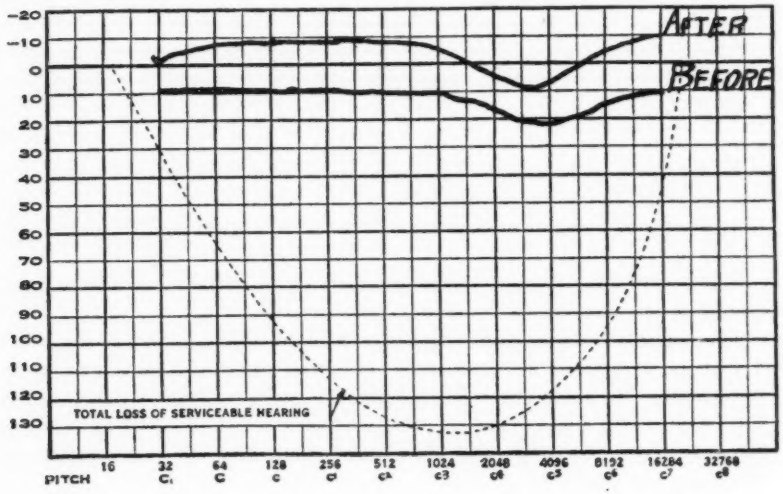
Rotation to left 10 times in 20 seconds. Nystagmus violent, duration 27 seconds

Rotation to right 10 times in 10 seconds. Past-pointing right hand 18 inches to right, left hand 18 inches to right

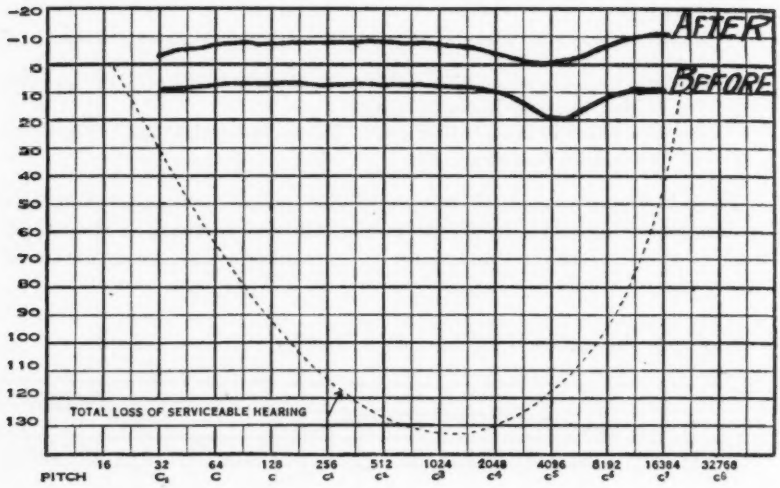
Rotation to left 10 times in 10 seconds. Past-pointing right hand 18 inches to left, left hand 18 inches to left

From these data obtained from vestibular tests it is seen that distention of the gastro-intestinal tract produces *a*, marked increase in the amplitude of after-nystagmus, *b*, increase in duration of after-nystagmus by 30 per cent, and *c*, increase in past-pointing by 350 per cent.

B. *Cochlear.* Audiogram of left ear. April 30, 1927.



Audiogram of right ear. April 30, 1927.



From these audiograms it is seen that, except for minor variations, distention of the gastro-intestinal tract produces raising of the auditory acuity over the entire tone scale by from 10 to 20 sensation units or by from 15 per cent to 25 per cent.

These experiments were repeated many times with essentially exactly similar results.

Discussion. Distention alone is sufficient to produce the response. The same experimental results were obtained whether food, air at room temperature, or hot water was used for distention. Thus neither thermal nor chemical change is necessary to produce the result. It is, of course, possible that during digestion other factors than distention may enter into the complete reaction.

It is not certain how the lowering of the threshold of the labyrinthine functions is accomplished. It is shown elsewhere (5) that gastro-intestinal distention produces marked vasomotor changes. We (6) have also shown that the character of the labyrinthine response after blood pressure changes is quite different from those we report here. Blood pressure changes affect only the upper tone scale whereas gastro-intestinal reflexes affect the entire tone scale. Therefore, distention of the gastro-intestinal tract affects the labyrinthine functions purely reflexly and not through changes in blood pressure.

SUMMARY AND CONCLUSIONS

Distention of the gastro-intestinal tract by food, air or hot water causes a lowering of the threshold for labyrinthine stimuli: auditory acuity is raised from 15 to 20 per cent, after-nystagmus becomes much more vigorous and increases 15 to 20 per cent in duration, and past-pointing may increase from 300 to 400 per cent.

Distention of the gastro-intestinal tract affects the labyrinthine function not through blood pressure changes but purely reflexly.

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WATER RETENTION UNDER LOW BAROMETRIC PRESSURE

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In 1898 Bouchard, by placing a fasting man on a self registering balance of Reider, observed that instead of obtaining a curve of loss in body weight declining continuously it would rise from time to time, indicating a gain in weight of 10 to 40 grams. These positive variations were purely temporary and were quickly followed by a further decrease. Experiments with dogs yielded similar results. Bouchard considered various possibilities in seeking an explanation of these temporary increases in weight but finally came to the conclusion that the incomplete oxidation of fat into glycogen is the only tenable explanation. He offers no explanation of why this incomplete oxidation of fat should take place periodically.

In 1904 Polimanti observed that in hibernating marmots there were periods when there was no loss in weight and occasionally there was an actual gain. The increase in weight is followed generally by an abrupt loss which is greater the larger the preceding gain. Polimanti ascribed these variations in the descending weight curve of the hibernating marmots to changes in barometric pressure.

Morgulis in discussing this said "of course there is no reason for assuming with Polimanti that the oscillations in body weight are somehow regulated by cosmic conditions, a low barometric pressure presumably tending to produce an increase in weight."

Various hypotheses have been offered to explain these temporary increases in weight.

1. That it is due to the hydroscopic properties of the animal's fur, but Polimanti showed that the variations appeared in the body weights of his marmots even though he kept them under different conditions of humidity.

2. That it is caused by a retention of either carbon dioxide or oxygen in the body. This could be occasioned by intermediary products of metabolism being formed that are not readily eliminated by the system. This would also explain, as Morgulis points out, the abrupt loss of weight following the increase, for there would be a rapid and augmented excretion as soon as the intermediary substances were oxidized to water and carbon

dioxide. This hypothesis involves the formation of glycogen from fat and its temporary storage in the body. In the equation

$$2(C_{57}H_{110}O_6) + 67O_2 = 16(C_6H_{10}O_5) + 18\ CO_2 + 30\ H_2O$$

fat glycogen

the weight of the fat used to that of the glycogen produced is about 1 to 1.4. This seems a very plausible hypothesis, for Valentin and others have shown there is a new formation and storage of glycogen in the liver of hibernating animals.

3. That there is water retention in the body of animals during low barometric pressure.

Hasselbach and Lindhard found in a study of high climates that there was a decreased urine excretion under low barometric pressure and that the animals soon became acclimated to the low pressures. But they gave no data as to food and water intake.

Holck observed that on a relatively uniform diet, water intake and daily activity there were wide variations in the daily quantity of urine excreted, a smaller amount being excreted, he believed, on days of low barometric pressure.

It was thought that it would be valuable to determine 1, whether animals under low barometric pressure actually excrete less urine than their water intake; 2, whether there is a greater amount of water than normal stored in the tissues of animals that have just been exposed to low barometric pressure.

Method. Dogs were used to determine the first point and rats the second. An airtight tank, such as described by Kolls and Loevenhart, connected to a motor driven air pump and equipped with electric and hand operated valves was used. Arrangement was also made so that the incoming air could be bubbled through water and thereby be saturated with moisture.

Small dogs were selected for the experiments and a cage was built inside the tank, arranged for collecting urine. For drinking purposes a measured amount of water was placed in a beaker that fit snugly in a container on the side of the cage, and to measure the evaporation another vessel with the same diameter was placed in the tank under the cage and a measured quantity of water put in it.

Female dogs were used and they were catheterized before each experiment and at the end of it in order to obtain the exact quantity of urine formed during the 24-hour test.

The dogs were fed on a uniform diet, having been on this diet in each case for several days before each experiment was run.

Usually the tests were run as follows: The dogs were placed in the tank, after the urine collecting bottle, the water beaker and evaporating dish were in place, the door closed and the air pump started with the valves

open so that the pressure would not be lowered. Barometric pressure and temperature was noted and observations were made at about every two hours to see that conditions were kept as nearly uniform as possible. After 24 hours the dog was taken out for one hour, the cage cleaned, the water intake and urine measured, the amount of evaporation determined, and after the cage was prepared as before the dog was placed in the tank again, the valves set so as to reduce the barometric pressure in the tank any desired amount as is shown in the data below, the electrically controlled valve opening when the mercury in the manometer made contact with a nickel wire which was placed at the desired level. At the close of the 24 hours under low barometric pressure the dog was usually allowed to rest 24 hours and then the conditions of the experiment reversed, that is, the low barometric pressure test being taken first and the control second.

However, the tests were sometimes taken continuously as described above for three days and nights with a test under low barometric pressure preceded and followed by 24-hour control periods, and on one occasion the animal was kept in the tank, with one hour intermission out of every 25 hours, for 4 days and nights, alternating periods of normal pressure with low pressure. Careful observations were made as to temperature and barometric pressures almost hourly during the course not only of this test but of all those made.

As an example of the way the tests were conducted and the typical results obtained, the following protocol is quoted:

December 8–9—9 a.m. Placed dog in tank; same food having been fed as previously—230 grams hamburger meat; 30 grams bone meal; 40 grams bread.

TIME	BAROMETRIC READING	TEMPERATURE	NEGATIVE PRESSURE
		°C.	cm. Hg
9 a.m.	757	19	0
10 a.m.			1.3
11 a.m.			2
12 m.	757	19	2.8
1 p.m.			3.7
2:30 p.m.			4.8
3:30 p.m.	755	16	5.4
5 p.m.	755	16	5.6
11:30 p.m.	754	19	5.6
6:30 a.m.	753	18	5.6

	cc.
Urine excreted	70
Water drunk (net)	140

On the day following, i.e., December 9–10, with the dog under the same conditions in every way that it was possible to control with the one condition of no change in barometric pressure, excreted 163 cc. of urine and drank 98 cc. of water.

Thus during this 48 hour period there was apparently a retention of 70 cc. of water in the body of the dog during the 24 hours under low barometric pressure and then during the 24 hour period under normal conditions of pressure, there was a loss of 65 cc. of water greater than the intake.

In the following table all the results that are comparable are used, those being eliminated where known factors have influenced the results obtained; for example, on a few occasions the dog developed diarrhea and the results obtained either as far as urine collected or water consumed would have indicated in no way the actual water balance of the body.

On other occasions the barometric pressure was not kept constant because of mechanical troubles and the results not only of the test but of the control are not reported.

Water balance of dog in tank

Control

DATE	NET WATER INTAKE	URINE EXCRETED	WATER BALANCE
	cc.		*cc.*
November 23–24	155	167	−12
December 5– 6	130	165	−35
December 7– 8	77	178	−103
December 9–10	98	163	−65
December 11–12	173	230	−57
December 14–15	147	210	−63
December 16–17	150	103	+47
December 20–21	187	182	+5
January 3–4	200	166	+34
March 12–13	40	75	−35
June 14–15	14	260	−246
June 18–19	62	72	−10
June 22–23	70	80	−10
June 26–27	145	230	−85

Negative pressure

DATE	NET WATER INTAKE	URINE EXCRETED	WATER BALANCE	NEGATIVE PRESSURE
	cc.			*cm. Hg*
November 24–25	69	59	+10	5.6
December 6– 7	230	223	+7	5.6
December 8– 9	140	70	+70	5.6
December 10–11	315	193	+122	5.6
December 15–16	265	226	+39	5.6
December 19–20	257	178	+79	9.6
January 2–3	273	171	+102	9.8
March 13–14	40	50	−10	9.5
June 17–18	70	120	−50	2.6
June 20–21	145	65	+80	5.0
June 25–26	90	65	+25	9.0

DISCUSSION. In this table there are 14 control tests and eleven tests made under negative pressure. These tests were made alternately as nearly as it was possible, the reason for the larger number of controls being that on two occasions controls were made both before and after the test.

In eleven cases in the control tests the water intake was less than the urine excreted while in three cases the reverse was true. The average net loss of water per day for the body during these 14 days was 45.3 cc. This net loss possibly seems greater than the evidence would justify for on June 14–15 (see table) the intake was only 14 cc. while the excretion was 260 cc., which is obviously an exceptional case, the dog having probably drunk considerable water before being put in the tank. If, however, this case be left out of consideration entirely there is still a net loss per day of almost 30 cc.

In the tests under negative pressure there were nine days when the water intake was greater than the water elimination, and two days when the reverse was true, showing a net average of water retention for the eleven days of 43.1 cc. per day.

As to whether the water retention under low pressure is proportional to the negative pressure it would be difficult to say from the data, there being so many other factors that might influence the water intake as well as the urine formation, these variations being so great that a slight change in barometric pressure might be offset by other factors. Hence attempts were only made to reduce the pressure to an amount that should be sufficient to offset any normal barometric change and yet not low enough to produce any complications due to lack of adequate oxygen pressure.

In the earlier experiments of this series there was no attempt made to saturate the air that was drawn through the tank with moisture. Inasmuch as it was thought that evaporation from the dog's lungs might be an important factor in the water output, arrangements were made to bubble all the air that was drawn into the tank through water and in this way insure its saturation to a degree that would make evaporation from the lungs negligible. The air drawn through in this way was so nearly saturated that the dish of water placed in the tank under the cage in order that the evaporation taking place on a known surface might be measured showed no measurable loss.

TESTS WITH RATS. In order to determine whether animals that have been under low barometric pressure have more water in their tissues than they normally have, 40 rats were selected and they were divided into groups of five. Each group was fed upon a diet of 65 grams of Hamburger steak, 35 grams of bread and 10 cc. whole milk per day. These rats were almost exclusively males, there being only 3 females, apparently non-pregnant. Care was taken to match each rat in each control test with one of almost the same size, apparent age, weight, etc.

The method procedure consisted in placing one group in the tank and keeping them there from 12 to 48 hours for control, or for the test, removing and killing quickly, skinning each rat and grinding up the remaining tissue in a meat grinder, catching the ground tissue in an evaporating dish. At first this tissue was sampled and a definite amount was desiccated; later all the tissue was desiccated and the percentage of loss calculated.

The data on the first test and control follow; the remainder will be given only in summary.

WEIGHT OF RAT	WEIGHT OF TISSUE	LOSS OF WEIGHT
January 13. Control. 24 hours in tank		
grams	*grams*	*grams-per cent*
172	100	68.65
132	100	67.60
176	100	68.20
164	100	65.10
149	100	67.75
Total loss		337.3 grams
Average loss		67.46 grams and per cent
January 14. Test under 9.4 cm. Hg. Negative pressure for 24 hours		
165	100	67.50
157	100	68.40
132	100	68.20
169	100	68.15
150	100	68.55
Total loss		340.8 grams
Average loss		68.16 grams and per cent

Here we have a very slight difference in water content of the tissues. The 500 grams of rat tissue that had been under low barometric pressure containing 3.5 grams more water than the 500 grams that had not been subjected to this low barometric pressure. This is true of all the other tests made if the whole is considered but there are individual exceptions.

Summary of results on 40 rats

20 CONTROLS		20 LOW BAROMETRIC PRESSURE	
Weight of tissue	Loss of weight	Weight of tissue	Loss of weight
grams	*grams*	*grams*	*grams*
500.0	337.30	500.0	340.8
672.5	475.00	754.0	533.7
876.8	581.25	986.1	664.0
870.6	581.20	1141.6	763.9
2919.9	1974.75	3381.7	2302.4
Loss 67.63 per cent		Loss 68.04 per cent	

This shows an apparent water retention of only 0.41 per cent or 4.1 grams per kilogram body weight. This water retention is well within the experimental error and although there was a slightly greater water content in the total tissues of each group of rats that had been subjected to low barometric pressure than those of the control tests, there were individual rats where the reverse was true.

If there is water retention in the rats that were kept under low barometric pressure, there were two conditions of the experiment that might tend to minimize the results. 1. The necessity of skinning the rats in order that they might be ground. 2. In selecting the rats for each control and for each test wherever there was a choice necessary to be made between the apparently well-nourished rat and one that did not look so, the former was placed with the control rats so as not to produce apparent water retention due to any other known cause than the change in barometric pressure. The results obtained were in spite of these precautions.

Total activity. By means of pieces of inner tube connected to a manometer the total activity for 24-hour periods of a dog in the tank at various barometric pressures was taken. By using bromoform and a long manometer with pressure inside the inner tube adjusted properly the apparatus could be made sufficiently sensitive to record the total activity even to the respiratory movements of the dog when it was lying down.

The following was observed in regard to the total activity of the dog in the tank; the first 24 hours the dog was more active than the second 24 hours regardless of whether the low pressure test or the control was run first. However, there was evidence of greater activity during the low pressure periods than during the control period when tracings were compared with one another in such ways as to eliminate the effect of this time element. To accomplish this tracings that were made during the first 24 hours with the animal under low pressure were compared with tracings made during control periods that were also taken immediately after the dog had had a period of rest outside the tank.

It is suggested that this restlessness is caused by the retention of water in the body and is not the cause of it, for while it is possible that increased activity might increase the metabolites in the body and hence cause a change in water balance, on the other hand, hypotonicity of the body fluids might act as a primary stimulus, as Carlson found was the case with the Limulus.

The restlessness of various animals that is so often noted before a storm might be accounted for by the low barometric pressure and the consequent water retention. With an animal that is particularly sensitive to this condition there is thus given a fore knowledge of approaching bad weather so that migratory birds are away before there is any sign in the sky of a weather change. Hogs usually bed themselves down and cattle on the

range become restless and seek shelter hours before there is any evidence of impending change in reaction that will impress the senses of man.

Cameron and Carmichael observed that a sudden fall in barometric pressure induced tetany in thyroid fed rats.

In the case of people who suffer with rheumatism or any one of several other affections that make the nerves particularly sensitive, it is often claimed that they can tell of the approach of bad weather because of increased pain or that they "feel it in their bones." There is undoubtedly a periodicity to their sensitiveness to a condition that possibly makes them observe any slight change.

CONCLUSIONS

1. There is water retention in the bodies of dogs and rats when the barometric pressure is lowered 2.6 to 9.8 cm. of mercury during a period of 12 to 48 hours.

2. This disturbance of the water balance of the body is accompanied by restlessness.

3. The possibility of this hydration being the mechanism inducing the reactions on the part of many animals and some people of approaching weather changes is discussed.

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THE EFFECT OF YEAST FEEDING ON SOME BLOOD CONSTITUENTS OF HENS

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The extensive use of yeast in various forms by mankind as a nutrient as well as a food for animals suggested the importance of studies on yeast as a constituent of the diet. As the advantages and disadvantages of a food become more evident if given in large amounts, and as the composition of the blood reflects the effect of the food on the organism, the writer undertook the following experiments of feeding hens large quantities of yeast, either alone or in addition to other ingredients.

According to Meisenheimer (23), as much as 8 per cent of the total nitrogen of yeast is in the form of purine bodies (guanin and adenin). A yeast diet may therefore cause a rise in the uric acid of the blood, as was shown experimentally by Funk, Lyle and McCaskey (7) on men.

As active yeast contains a series of powerful nucleases, it seemed of value to study the resulting differences in the blood composition due to this factor on high and low purine diets. Up to the present no attempt has been made to lower "physiologically" the per cent of purine bodies in the food by inhibiting the liberation of uric acid from nuclein bodies during the process of absorption and utilization, or by creating within the body such conditions as would stimulate the synthetic power of the corresponding nucleases to store the uric acid in the form of nuclein bodies. The purpose of the writer was to study the possibilities also in this direction.[1]

Hens were chosen as material for the experiments because of the ease of forced feeding and also because in the hen "the uric acid is all free and in the plasma" (Jones, 16). Besides, Folin and collaborators (4) said that in birds "the uric acid in the muscles bears no relation to the uric acid content of the circulating blood." Another reason was the high resistance of birds toward uric acid intoxication. In the blood of ducks with ligated ureters Folin and collaborators (4) obtained, e.g., up to 400 mgm. per cent of uric acid. Of interest is also the well-known double origin of uric acid in birds—by synthesis and by derivation from purine bodies. Kionka and

[1] Since 1923, following the suggestion of Dr. Franklin McLean of the Peking Union Medical College, the writer has undertaken the study of the uric acid problem.

Bannes (cited by Hutyra and Marek, 14) produced typical gout in birds by continuous feeding with horse flesh, which is rich in hypoxanthine. Recently Naito and Nishioka (25) found that the human organism also is capable of synthesizing uric acid from urea. Hens are known to respond to a high purine diet in the same way as mammals. It was hoped that studies on hens might help in the understanding of some obscure chapters in the uric acid problem of mammals.

MATERIALS AND PROCEDURE. Eighteen normal hens, Barred Plymouth Rock, weighing about 5 pounds, from 6 to 9 months old, hatched at the same time and coming from one stock, were fed the following diets:

Two hens (*nos. 6 and 7*)—semi-polished rice (a low purine diet) and water ad libitum.

Three hens (*nos. 8, 9 and 10*)—semi-polished rice and water during the first period, and semi-polished rice and diluted liquid Taka-Diastase during the second period.

Two hens (*nos. 11 and 12*)—inactive yeast (heated, Fleischmann's).

Two hens (*nos. 13 and 14*)—inactive yeast and powdered Taka-Diastase.

Three hens (*nos. 15, 16 and 17*)—active yeast (Fleischmann's).

One hen (*no. 18*)—active yeast and wheat bran.

One hen (*no. 19*)—active yeast and wheat bran the first period, and active yeast and phytin the second period.

Two hens (*nos. 20 and 21*)—active yeast the first period, and active yeast + acid sodium phosphate[2] the second period.

Two hens (*nos. 22 and 23*)—active yeast the first period, active yeast + acid sodium phosphate[2] the second period, and active yeast + acid sodium phosphate[2] + cornstarch the third period.

In addition, *five hens* (*nos. 1, 2, 3, 4 and 5*) were fed a standard diet and served as controls.

All the hens looked healthy and strong. The droppings were mostly more or less soft.

The blood for analysis, about 40 cc., was taken at approximately the same hour after feeding. It was drawn only once from each hen from the jugular vein in oxalated tubes, centrifuged at once, and the plasma analyzed for glucose (Folin and Wu's method), chlorine (Whitehorn's method), nonprotein nitrogen (Folin and Wu's method), uric acid (Benedict's method), creatinine (Folin and Wu's method), cholesterol (Bloor's method), inorganic phosphorus (Brigg's method), and albumin and globulin (Wu and Ling's method, 39). Details can be found in tables 1, 2 and 3.

In order to determine the presence of a nucleosidase in active yeast and Taka-Diastase, and also to determine the amount of uric acid present in

[2] The acid sodium phosphate was given in daily doses of 0.1 gram per kilo of body weight of the hens, as such doses were found by Salvesen, Hastings and McIntosh (33) to be harmless even if given over a long period.

inactive yeast (Fleischmann's) and Taka-Diastase, corresponding determinations were done in connection with *in vitro* experiments, as suggested by Dr. P. A. Levene of this Institute. The findings are given in table 4.

RESULTS. The data are given in tables 2, 3 and 4. Table 1 gives the controls. Hens 11 and 12, fed inactive yeast, show a higher albumin content than globulin. Hens 13 and 14, fed inactive yeast and Taka-Diastase, show higher globulin figures than albumin. In normal hens 1, 2, 3, 4 and 5, the per cent of globulin surpasses that of albumin. The non-protein nitrogen is high in hens 13 and 14 fed inactive yeast and Taka-Diastase. If the corresponding figures for uric acid nitrogen $\left(=\frac{uric\ acid}{3}\right)$ are subtracted from the non-protein nitrogen figures, the residual non-protein fractions remain nevertheless higher than the corresponding figures in the controls.

TABLE 1

Blood plasma of normal hens (controls) fed a standard diet (per 100 cc.)*

NUMBER	SUGAR	N.P.N.	URIC ACID	CREATININE	ALBUMIN	SERUM GLOBULIN	CHOLESTEROL	CHLORINE	INORGANIC PHOSPHORUS
	mgm.	*mgm.*	*mgm.*	*mgm.*	*gram*	*grams*	*mgm.*	*mgm.*	*mgm.*
1	266.6	16.06	6.85	1.11	1.96	2.04	94.7	434	4.75
2	285.7	18.75	6.33	1.00	1.57	2.11	97.1	432	4.60
3	307.7	15.80	6.09	0.95	1.88	2.00	91.3	460	5.10
4	330.5	24.01	6.13	1.07	1.46	1.76	115.3	447	4.24
5	333.0	21.42	7.24	1.11	1.46	2.31	115.0	460	4.44
Average	304.7	19.21	6.53	1.05	1.67	2.04	102.7	447	4.63

* Grain (corn, oats, wheat)
Mash (beef scrap, bran, middlings, corn meal) } ad libitum.
Oyster shell, grit, charcoal

The inorganic phosphorus runs unusually high in hen 19, fed active yeast and phytin, and unusually low in hens 11 and 12, fed inactive yeast. The low phosphorus of hen 10 (fed semi-polished rice and Taka-Diastase) has to be noted too. The figures for the uric acid content of plasma of our hens vary largely, depending upon the diet.

Table 4 shows that active yeast as well as Taka-Diastase is capable of liberating uric acid from nuclein bodies.

DISCUSSION. *Adequacy of the yeast diet.* According to Lippincott (21), the maintenance standard per day for a hen of 5 pounds weight consists of 9 grams of digestible protein, 45 grams of digestible carbohydrates, and 4.5 grams of digestible fat. According to this author brewers' grains (barley refuse from the brewing of alcoholic beverages, a large portion of which is yeast) make an excellent food for hens. The analysis of Fleisch-

TABLE 2

Blood plasma of hens fed semi-polished rice, inactive yeast and Taka-Diastase (per 100 cc.)

NUMBER	S.P. RICE*	INACTIVE YEAST†	TAKA-DIASTASE	SUGAR	N.P.N.	URIC ACID	CREATININE	ALBUMIN	SERUM GLOBULIN	CHOLESTEROL	CHLORINE	INORGANIC PHOSPHORUS
				mgm.	*mgm.*	*mgm.*	*mgm.*	*gm.*	*gm.*	*mgm.*	*mgm.*	*mgm.*
6	27 days	—	—	235.2	16.22	6.16	1.03	2.25	1.72	150.9	411	4.54
7	Same	—	—	286.0	17.14	4.71	1.00	2.07	2.20	107.3	414	3.70
8	35 days	—	Liquid T. D.‡ for last 8 days of rice feeding	224.2	15.51	5.60	1.18	1.83	2.46	80.9	406	4.14
9	Same	—	Same	258.7	19.80	12.54	1.13	2.06	1.76	107.4	426	5.23
10	Same	—	Same	231.4	19.75	8.01	1.13	2.07	2.38	136.4	437	2.29
11	—	7 days	—	250.3	18.75	8.06	1.30	1.96	1.40	84.1	400	2.17
12	—	8 days	—	250.0	22.70	10.66	1.07	2.15	2.03	88.2	398	3.22
13	—	7 days	Powdered T. D.§ for last 6 days of yeast feeding	250.7	33.33	16.12	1.25	1.65	2.78	100.7	412	5.26
14	—	Same	Same	242.1	30.00	13.33	1.15	1.73	2.53	87.6	401	4.17

* Ad libitum.

† 100 grams of dry yeast, moistened to form a paste, per capita per day by cramming (in two portions).

‡ Of Parke, Davis & Co. (liquid 4 per cent) diluted with 4 parts of water (0.8 per cent). Given ad libitum. Drinking water removed.

§ Of Parke, Davis & Co. (powder). Given in gelatinous capsules before yeast was fed. Average daily dose: 2.4 to 3.6 grams.

TABLE 3

Blood plasma of hens fed active yeast, wheat bran, phytin, starch and NaH_2PO_4 (per 100 cc.)

NUMBER	ACTIVE YEAST	WHEAT BRAN	PHYTIN	STARCH	NaH_2PO_4	SUGAR	N.P.N.	URIC ACID	CREATININE	ALBUMIN	SERUM GLOBULIN	CHOLESTEROL	CHLORINE	INORGANIC PHOSPHORUS
						mgm.	*mgm.*	*mgm.*	*mgm.*	*gm.*	*gm.*	*mgm.*	*mgm.*	*mgm.*
15	7 days*	—	—	—	—	250.6	23.07	13.35	1.03	1.74	3.80	72.5	404	5.17
16	14 days*	—	—	—	—	249.1	30.00	20.00	1.00	1.76	2.44	88.2	411	5.40
17	21 days*	—	—	—	—	296.3	23.24	14.02	1.07	1.91	2.50	93.8	396	5.33
18	13 days	13 days†	—	—	—	266.0	24.22	10.03	1.00	2.07	1.90	94.0	440	5.53
19	Same*	9 days†	Last 4 days of yeast feeding‡	—	—	400.0	35.31	17.10	1.11	1.86	1.95	75.2	397	7.90
20	18 days*	—	—	—	Last 8 days of yeast feeding§	250.0	35.29	15.27	0.94	2.16	2.97	187.5	336	4.84
21	Same*	—	—	—	Same	322.4	27.27	12.64	0.94	2.13	2.72	88.2	378	4.68
22	25 days*	—	—	Last 10 days of yeast feeding¶	Last 15 days of yeast feeding§	286.3	25.02	17.15	1.07	1.55	2.01	100.0	386	4.59
23	Same*	—	—	Same	Same	251.1	18.75	10.00	1.07	1.69	2.37	95.3	397	5.26

* About 90 grams of dry yeast, moistened to form a paste, per capita per day, by cramming in two portions.

† Yeast and bran were mixed in equal parts, moistened, and fed ad libitum. About 100 to 150 grams of this mixture were eaten per day.

‡ Bran was excluded. Phytin (in tablets, 3 grams daily dose) was given instead before yeast was fed (1.5 gram each time).

§ 5 cc. per capita per day of a solution ($NaH_2PO_4 \cdot H_2O$—4.45 grams; H_2O—to 25 cc.) given in two portions mixed with the yeast (5 cc. of this solution contain 0.2 gram of P).

¶ About 20 grams of starch per capita per day, mixed with the yeast, phosphates and water to form a paste. Fed by cramming.

mann's dry yeast, performed by Hawk, Smith and Holder (8) showed the yeast to contain 52.41 per cent of nitrogenous substance, 37.13 per cent of carbohydrates, and 1.72 per cent of fat. According to Völtz (36) the protein of yeast is digested by animals to an extent of 88 per cent and the nitrogen-free extractives to 100 per cent. Osborne and Mendel (28) give for the yeast protein a digestibility (in rats) of 74 to 83 per cent.

Osborne and Mendel (28) and Meisenheimer (23) consider that the nitrogenous substances of yeast are only about 50 per cent in the form of protein, the remaining part being mostly amino acids, purine bodies (8 per

TABLE 4

The amount of uric acid liberated in yeast autolysate and in a lysate of inactive yeast + Taka-Diastase (calculated per 100 grams of the initial dry substance)*

TIME OF URIC ACID DETERMINATION	COMPOSITION OF SUSPENSIONS			
	Active yeast...100 gm. Chloroform water to.....1 liter Toluene.....1 cc.†	Inactive yeast..100 gm. Taka-Diastase. 10 gm. Chloroform water to.....1 liter Toluene.....1 cc.†	Inactive yeast...1 gm. H_2O.....to 10 cc. Boiled at once	Taka-Diastase..1 gm. H_2O.....to 10 cc. Boiled at once
	mgm.	*mgm.*	*mgm.*	*mgm.*
At once	—	—	26.6	40.0
24 hours	30.8	32.0	—	—
48 hours	32.0	34.8	—	—
3 days	30.8	—	—	—
4 days	—	33.3	—	—
5 days	30.8	—	—	—
8 days	—	36.4	—	—
9 days	29.6	—	—	—

* Determined by Benedict's method in the following way: To 10 cc. of the thoroughly mixed suspension were added: 8 cc. H_2O, 1 cc. Na_2WO_4 (10 per cent) and 1 cc. H_2SO_4 (2/3N). After mixing and keeping the suspension for 20 minutes it was filtered and 1 cc. of the filtrate taken for the determination of uric acid. Four extra cubic centimeters of H_2O had to be added to it in order to avoid turbidity.

† The suspension was shaken from time to time and kept in a stoppered flask at room temperature.

cent), and ammonia. The actual protein content of yeast may therefore be taken as 25 per cent, but the amino acids may also be utilized by the organism for the synthesis of the body proteins.

According to Völtz (36), yeast contains all the nitrogenous substances which are required for all the physiological functions of the animal organism. Osborne and Mendel (28) "have kept rats successfully for more than a year, covering the period of growth, upon a diet in which yeast furnished the sole source of nitrogen as well as water-soluble vitamine." Fleischmann's inactive (heated) yeast which was used in our experiments still contains the water-soluble vitamin.

Therefore the 90 to 100 grams of dry yeast fed to the hens in the present experiments were more than adequate in protein and number of calories (although the ratio protein:carbohydrates was high). The observed changes in the blood composition of the experimental hens can therefore not be attributed to any stage of starvation.

Phosphorus. According to Hawk, Smith and Holder (8), Fleischmann's dry yeast contained 8.75 per cent of ash, 54.5 per cent of which was P_2O_5. It could be expected that such an enormous amount of phosphorus, mostly in the form of nucleic acid, would have greatly influenced the inorganic phosphorus content of the plasma of the experimental hens fed a yeast diet. But the data obtained on hens 15, 16 and 17, fed on active yeast, show only a slight rise in the inorganic phosphorus content of the blood. According to Levene and Medigreceanu (20), when treated with the extract of intestinal mucosa, "the yeast nucleic molecule decomposes into the following substances: phosphoric acid, purin bases, d-ribose, cytidin and uridin." It has been also recorded by many observers that the yeast nucleins suffer during the process of autolysis a complete disintegration with liberation of phosphoric acid and of free purine and pyrimidin bases. Therefore, the ingested active yeast supplies the organism with a large amount of inorganic phosphorus, the fate of which may vary.

It is of interest to note that hens 20, 21 and 22 show a much lower inorganic phosphorus content in the plasma, in spite of the same diet, consisting of active yeast, and an extra addition of phosphorus in the form of sodium diphosphate. The acid sodium phosphate might have inhibited, in our case, the action of the nucleases of the intestinal mucosa and the yeast on the nucleic acid of yeast by changing the pH (20) and as a result no phosphoric acid, or an insignificant amount, was set free. Hen 23 showed, under similar conditions, a higher inorganic phosphorus in the plasma, which is possibly due to the activity of the yeast itself in the intestinal tract resulting in the utilization of the acid sodium phosphate by the yeast cells in a medium containing starch.

At the present time very little is known about the rôle in the organism of the specific enzymes (nucleases) ingested with the food. Active or inactive (heated) yeast is fed to animals and prescribed by physicians without knowing exactly how it is going to affect the organism in toto. Our experiments with hens 11 and 12 show that when they are fed a sole diet of inactive yeast the inorganic phosphorus of the plasma drops to the abnormally low figures of 2.17 to 3.22 mgm. per cent in spite of the fact that inactive yeast still contains the same 4.4 per cent of P_2O_5 as the active one. But when Taka-Diastase[3] is added to this diet, the figures for inorganic phosphorus in the plasma of hens 13 and 14 rise to 4.17 to 5.26 mgm. per

[3] Taka-Diastase is the patented name of a ferment, manufactured from the *Aspergillus orizae* mould, which is largely used in China and Japan as a yeast.

cent. Taka-Diastase contains a large number of enzymes, among which are nucleases capable of liberating the phosphoric and uric acid precursors from yeast nucleic acid, as was shown by Iwanoff (15) for the mould *Aspergillus niger* and by the writer for Taka-Diastase (see table 4). These experiments are interesting as they demonstrate the difference in the resulting effect on phosphorus metabolism between the body nucleases of the digestive tract and the foreign nucleases ingested.

Plasma proteins. Winterstein (38) gives for normal chicken blood serum the albumin:globulin ratio to be <1 (analyzed by the $MgSO_4$ method). By Robertson's (31) refractometric method this ratio is >1. Our experiments show that the effect of the enzymes of yeast and Taka-Diastase on the blood is not limited exclusively to the phosphate fraction. The plasma of hens 11 and 12, fed inactive yeast, shows the ratio albumin: globulin to be >1. The same ratio in hens 13 and 14, fed in addition Taka-Diastase, is <1, as is also the case in hens 15, 16 and 17, fed active yeast, and in the controls 1, 2, 3, 4 and 5. This difference can perhaps be explained by the absence of enzymes in the food of hens 11 and 12 and the presence of such in the food of the other groups. Enzymes are believed to be of protein nature, and as shown by Horvath and Chang (10) for lipase and by Pfeiffer and Standenath (30) for peptidases, they possess the capacity of penetrating through the intestinal wall into the blood stream. The writer made no direct attempt to prove the penetration of Taka-Diastase through the intestinal wall by measuring the diastatic power of the blood because Kito (17) showed that in this respect there is no difference even after the direct injection of Taka-Diastase into the blood stream. But in an indirect way the absorption of the nuclease of Taka-Diastase from the intestines was clearly demonstrated in hens 9 and 10 (see table 3) by a rise in the uric acid content of the blood on a low purine diet (of semi-polished rice) with the addition of Taka-Diastase. As the "extra" uric acid could not be derived in these hens from the food, it means that the nuclease of Taka-Diastase had penetrated through the intestinal wall and acted upon some nuclein bodies of the organism in the tissues or the blood. The semi-polished rice contains a practically negligible amount of purine bodies, and 100 cc. of the 0.8 per cent Taka-Diastase solution contained only 3.2 mgm. of uric acid. According to Winterstein (38), the amount of blood in hens is from 3.9 to 8.7 per cent. If we take the lowest percentage (3.9) as a basis for calculation, it will show 87.7 grams of blood in a 5-pound hen. It is evident that 3.2 mgm. of uric acid are not capable of raising to a considerable extent the uric acid level in 87.7 grams of blood.

Löhr and Löhr (22) and others have found a relative increase of serum globulin after injection of protein. Foreign enzymes, as being of protein nature, may perhaps, after having penetrated into the blood from the in-

testines, also be capable of causing a similar rise in the globulin fraction of the blood serum, as is the case in our experiments. Immune bodies, which are believed by some authors to be of enzymatic nature, also possess the capacity of penetrating from the intestinal tract and exerting their effect on the serum globulin, as shown by T. Smith, Howe and collaborators (34), (12), (13) to be the case for colostrum. The proteins of the food (albumins or globulins) are known to have no effect on the albumin:globulin ratio of the serum (37).

Uric acid. According to Levene and Medigreceanu (20), the intestinal juice is capable of forming mono-nucleotides from yeast nucleins, of which the purine nucleotides are further converted into nucleosides. The extract of the intestinal mucosa is capable of setting free the purine bases. But there is no proof that they are capable of raising the uric acid figures in the blood on a low purine diet. On the other hand, foreign nucleases (yeast nucleases and Taka-Diastase) seem to possess such a capacity.

It was just mentioned that the addition of Taka-Diastase to a low purine diet resulted in hens 9 and 10 in a marked rise in the uric acid content of the blood to 12.54 and 8.01 mgm. per cent, while our figures for the normal hen on a mixed diet were 6.53 mgm. per cent. This rise cannot be attributed either to starvation (see Lennox, 18, 19) or to avitaminosis because the hens were eating rice in sufficient quantities, the rice was only semi-polished, and, besides, even complete deficiency in water-soluble vitamin does not cause any rise in the blood uric acid. Folin's (5) data for mixed normal hens' blood are 4.9 mgm. per cent (determined by the indirect method). It was shown (see table 4) that Taka-Diastase contains powerful nucleases which possess the capacity of liberating uric acid from yeast nucleic acid. The rise in the blood uric acid following the ingestion of Taka-Diastase on a low purine diet can be interpreted as a liberation of uric acid from some purine compounds of the body. This effect of Taka-Diastase may perhaps be enhanced by some amino acids present in this diastase: Lennox (18) showed that feeding asparagine (during fasting) resulted in increased elimination of uric acid without any decrease in the level of circulating uric acid. The extra uric acid came therefore from the tissues. Perhaps amino acids are capable of exerting some effect on some nuclein bodies of the tissues similar to that of an enzyme. This point of view is supported by analogous findings of Falk and Nelson (2) for lipolytic activity that amino acids are capable of hydrolyzing esters.

The observed rise in the blood uric acid of our rice-Taka-Diastase hens means that the corresponding ferment (Taka-Diastase) is capable of penetrating through the intestinal wall. This interpretation may be applied also to yeast nucleases. Hens 15, 16 and 17, of table 3, fed on active yeast, show high uric acid figures in the blood plasma (from two to three times

the normal controls). The rise in the uric acid content cannot be attributed in this case to the high protein content of yeast, because, according to Folin and collaborators (4), "A high protein diet reduces the circulating level of uric acid below the levels obtainable on low protein diet."

The data for a diet of inactive (heated) yeast (hens 11 and 12, table 2) and for inactive yeast plus Taka-Diastase (hens 13 and 14) show clearly that inactive yeast causes a smaller rise in the per cent of uric acid of the blood than inactive yeast plus Taka-Diastase. It is therefore remarkable that inactive yeast, which contains the same amount of nucleins and purine bodies as active yeast, is acting physiologically as a lower purine food. Biberfeld and Schmid (1) are persuaded that free purine bases and uric acid can hardly be absorbed in large amounts from the intestinal tract. These authors quote Abderhalden and Schittenhelm, who found that the nucleic acids become in the intestines very soluble and dialysable (mono-nucleotides and nucleosides) and are evidently absorbed as such. Hence the high uric acid content in our active yeast hens (or inactive yeast plus Taka-Diastase) might be attributed to the nucleases of yeast or Taka-Diastase absorbed from the intestines. The data for hens 15, 16 and 17 (table 3) show also that the uric acid figures are not dependent upon the length of the yeast feeding period (1, 2 or 3 weeks).

If these data, obtained on hens, would hold also for mammals, they would emphasize the enormous rôle played by "foreign" nucleases of the food, yeasts or bacteria on the plasma uric acid of other animals and man. Records of high percentages of uric acid will have to be reconsidered from this new point of view and the foods grouped not only according to their content of purine bodies (nucleins) but also in respect to the power of their nucleases. The differences in the data of various authors for uric acid of ox blood may be due partly to the enzymes of the feeds. Hutyra and Marek (14) give records of the capacity of corn smut (*Ustilago zeae*) to produce gout in hens (feeding). For cows corn smut is probably not very poisonous (Moore and T. Smith, cited by Pammel, 29). The diet of gouty individuals or of those predisposed to gout must be absolutely freed of such enzymes by cooking or other means of inactivating. The harm caused to the uric acid content by beer and wine may be due, from this point of view, chiefly to the presence of yeast nucleases. But the importance of the nucleases of the body tissues and organs must not be underestimated. Lennox (18) found that during fasting "under certain circumstances, marked changes in the concentration of uric acid in the blood were not accompanied by simultaneous and corresponding changes in uric acid output. There must be other factors (such as uricolysis or increased uric acid mobilization) to account for the facts observed." This mobilization was evidently caused by nucleases. The high blood uric acid, associated with leukemia, may be caused by the nucleases of the white blood cells.

It is also known that in man, "The uric acid concentration of the blood is a delicate, if not the most delicate index of renal function at our disposal" (Myers, 24). In nephritis the elevated figures for uric acid are perhaps due not only to uric acid retention but also to some liberation of kidney nucleases into the blood stream as the result of some sort of renal obstruction. In pancreatic obstruction, for example, the lipase is absorbed into the blood stream (9). The blood uric acid figures may perhaps be lower in double nephrectomy than in animals with ligated ureters.

As the action of every enzyme can be reversed, it was hoped that perhaps favorable conditions might be created in the body to synthesize nucleic acids taking the circulating uric acid as one of its constituents. Starling (35) says in his textbook of physiology that "the nucleases . . . . can certainly by synthesized by the animal." Attention must also be called to the fall in the non-protein nitrogen of the blood following the uric acid injection (Folin and collaborators, 4). It might be perhaps interpreted as a sign of some synthetic process where the uric acid and some N. P. N. constituents (amino acids?) are involved.

In order to study the possibilities of lowering the high uric acid of the blood plasma, due to active yeast feeding (continuing to feed approximately the same amount of yeast) substances rich in organic phosphorus (bran, phytin) or inorganic phosphorus (acid sodium phosphate) were given to a few hens. It was hoped that the presence of a large amount of phosphorus might inhibit the enzymatic decomposition of nucleic acid, as phosphoric acid is known to be one of its final products of cleavage. The carbohydrates of bran (starch, hexoses and pentosans), of phytin (inositol) and starch were added for the same reason. Yeast nucleic acids are known to contain a pentose (d-ribose) in their molecules, and the body nucleic acids d-ribose or a hexose.

The results were: Hen 18 (fed on active yeast and bran) showed a somewhat lower per cent of plasma uric acid, while in hen 19 (fed active yeast and phytin) the uric acid reached 17.10 mgm. per cent. Hens 20 and 21, where acid sodium phosphate was added, did not show any improvement in the uric acid content. So far the attempt has not been successful, but the work along this line will be continued.

Recently Neuberg and Kobel (27) found that amino-purines (guanin) are subject to decomposition in the presence of methylglyoxal. As the latter is a physiological product of catabolism of carbohydrates, the yeast feeding experiment with the addition of cornstarch and acid sodium phosphate was undertaken also for this reason. The results (hens 22 and 23) showed in one case a uric acid content of 17.15 mgm. per cent, and in the second 10.00 mgm. per cent. It is hard to say what caused the drop in the second case—a destruction of the purine ring in the intestines by bacteria (Rother, 32), the decomposition in the blood by methylglyoxal, or an

inhibition of the action of nucleases on nucleic acid by the presence of carbohydrates and phosphates.

In general high N. P. N. figures were obtained where nucleases were present in the diet. In the case of hens 13 and 14, the 3 grams of Taka-Diastase in the food contained only 18 mgm. of N.P.N., which could not have been responsible for the rise in the blood N.P.N. The high N.P.N. fraction may be due to the albumin fraction of yeast nucleoalbumins. In hens 20 and 21 the high figures for N.P.N.-uric acid are associated with very low creatinine figures. Inorganic phosphates are liberated during muscular work, and creatinine and purine bases are products of catabolism of the muscles. It is not impossible that there may exist some sparing effect of the inorganic phosphates on the destruction of more complex substances of muscular tissue. Recently Fiske and Subbarow (3) have offered proof of the presence in voluntary muscle of a compound containing one molecule each of creatine and phosphoric acid. During muscular contraction the compound evidently undergoes hydrolysis.

SUMMARY AND CONCLUSIONS

1. Active yeast and Taka-Diastase possess the capacity of decomposing nucleic acid with the liberation of uric acid.

2. Feeding hens active yeast results in an increase of the plasma inorganic phosphorus and uric acid.

3. Feeding hens inactive (heated) yeast causes a drop in the plasma inorganic phosphorus and a comparatively smaller rise in uric acid.

4. The addition of Taka-Diastase to an inactive yeast ration makes it, in so far as concerns the plasma inorganic phosphorus, uric acid, and protein fractions, similar to an active yeast ration.

5. The effect of a diet rich in nucleins on the inorganic phosphorus of blood plasma may depend also upon the presence of foreign nucleases in the intestines.

6. The effect on blood composition of the addition of phosphates and carbohydrates to an active yeast ration was also studied and discussed.

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COAGULATION TIME IN PARATHYROID TETANY

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Simpson and Rasmussen (1) reported an increase in the coagulation time following parathyroidectomy, but in 1916 (2) they rescinded their findings and reported no distinct effect on the coagulability of the blood after parathyroidectomy. They furthermore stated that in the dog, the blood coagulation time varied considerably in different animals and in the same animal from time to time. In both series of experiments, they used the graphic method of Cannon and Mendenhall (3) for estimating coagulation time. Dragstedt and Sudan (4) noted a marked reduction in the blood coagulability of dogs in parathyroid tetany, but stated that these findings were not constant.

Liu (5) has demonstrated that cod liver oil increased the amount of ionizable calcium in the blood, and the author (6) found cod liver oil to be beneficial in the treatment of parathyroid tetany. In parathyroid tetany diffusible calcium of the plasma is decreased, resulting indirectly from diminution of the organic compound (7). Since ionizable calcium plays an important part in the process of blood coagulation, clotting time should be longer than normal if this diffusible fraction is low.

For estimating clotting time the method of Lee and White (8) was found to be very satisfactory except more accurate and consistent results were obtained when 10 mm. tubes were used instead of 8 mm. This result may be due to greater capillary attraction in the latter. Four tubes, each calibrated to contain 1 cc. of blood, were used and the average time of coagulation for the four was taken as normal. The tubes and syringe were washed with soap and water and then rinsed with normal saline before using. The blood was drawn as quickly as possible from the external saphenous vein or heart and the time computed from the moment the blood was expelled into the test tubes until inversion of the tube to the horizontal plane caused no displacement. The average normal coagulation time varied from three to five minutes. The large percentage of normals averaged about four minutes. Consistent results were obtained and the normals varied only slightly from day to day thus differing from the findings reported by Simpson and Rasmussen (2) by using the method of Cannon and Mendenhall (3).

TABLE 1

DOG	NORMAL COAGULATION TIME	NORMAL SERUM CALCIUM	THYROPARA	TETANY	COAGULATION IN TETANY	CALCIUM IN TETANY	TREATMENT	AFTER RECOVERY	
								Blood coagulation	Blood calculated
	seconds	*mgm.*			*seconds*	*mgm.*		*seconds*	*mgm.*
A-1	4.5	10.0	March 30, 1928	Mild	7.8	6.8	Acterol	4.0	8.4
A-2	4.0	10.5	April 5, 1928	Severe	10.1	6.0	Acterol	5.0	9.75
A-3	3.4	9.7	April 19, 1928	Moderate	8.0	6.5	Acterol	Died from pneumonia	
A-4	3.3	9.8	April 19, 1928	Severe	7.0	5.5	Acterol	Died in tetany	
A-5	2.6	10.6	April 23, 1928	Severe	6.9	5.2	Acterol	3.9	10.0
A-6	3.0	9.9	April 27, 1928	Severe	6.0	4.9	Acterol	Died in tetany	
A-7	3.5	10.2	April 28, 1928	Severe	10.0	6.35	Acterol	4.5	9.0
A-L	2.5	11.0	April 23, 1928	Severe	5.5	6.1	Acterol	2.5	10.5
A-96	3.6	10.8	April 11, 1928	Moderate	8.1	6.1	Acterol	3.0	9.2
C-93	4.8	10.2	February 1, 1928	Very severe convulsions	Blood failed to clot in 30 hours		Cod liver oil	Died in tetany	
C-92	4.2	9.6	February 1, 1928	Severe	Blood clotted in 8 hours		Cod liver oil	3.8	8.8
C-91	3.9	10.0	November 19, 1927	Severe	9.3	4.9	Cod liver oil	Died from pneumonia	
C-89	3.4	9.0	November 11, 1927	Severe	15.2		Cod liver oil	3.7	8.6
C-87	3.8	9.8	November 1, 1927	Moderate	6.0	7.0	Cod liver oil	4.0	9.5
C-84	3.9	9.3	September 5, 1927	Moderate	8.5	7.0	Cod liver oil	3.6	9.0
C-83	3.5	9.7	August 26, 1927	Moderate	8.7	7.0	Cod liver oil	3.8	10.0
C-82	4.0	8.5	August 25, 1927	Moderate	7.25	6.70	Cod liver oil	Died in tetany	
C-81	4.5	9.0	June 30, 1927	Moderate	7.0	8.0	Cod liver oil	4.2	10.2

Blood coagulation time and an estimation of the serum calcium were made at frequent intervals after parathyroidectomy. With the onset of mild tetany there was usually a delay in coagulation time, and in severe tetany there was invariably a delayed coagulation time. The delay in clotting was greater sometimes when the animal was very depressed than when he showed muscle spasms and hyperpnea.

When the blood coagulability was decreased, the serum calcium was usually low (table 1). It would seem from a careful observation of many parathyroidectomized animals that the coagulation time is a better index to their true condition than is an estimation of serum calcium. In table 1 the longest delay in coagulation for each animal in tetany is given with the corresponding serum calcium value. It should be stated that this series of parathyroidectomized dogs was either given acterol[1] or cod liver oil[2] daily to aid in their recovery. They were given the regular stock diet of 400 to 500 cc. of milk, 130 to 150 grams of kibbled dog cake and in addition 250 grams of hamburger twice a week. The coagulation time for the animals manifesting mild to very severe tetany ranged from 5.5 minutes to 30 hours. All animals showing any degree of parathyroid tetany had a delayed coagulation time. The animal having a coagulation time of thirty hours had very severe tetanic convulsions and died in the attack. Neither cod liver oil nor acterol when given daily could prevent the fall in serum calcium or delay in coagulation time, but as reported elsewhere cod liver oil (6) and acterol (10) delayed the onset of tetany and ameliorated its severity. However, cod liver oil or acterol was efficacious in changing the coagulation time to normal in a period of two to four hours. The clotting time was again delayed in eighteen to twenty-four hours. After thirty to forty days of cod liver oil or fifteen to thirty of acterol administration the clotting time returned to normal as did the serum calcium.

Cod liver oil has been found effective in changing the delayed coagulation time of experimental obstructive jaundice to normal (9), and likewise, it changes a long coagulation time in parathyroid tetany to normal in two to four hours. On the reappearance of tetany coagulation time is again delayed. In a few cases, blood coagulation time was very long but returned to normal in two to four hours after the administration of 30 cc. of cod liver oil. The blood in one animal that was in severe tetany did not clot for eight hours. One ounce of cod liver oil was given by stomach tube and in two and one-half hours, the blood clotted in 3.8 minutes. This was repeated three times and the coagulation time was practically the same.

[1] Acterol was kindly supplied by Mead, Johnson & Co.
[2] Cod liver oil was supplied by Eli Lilly & Company.

SUMMARY

Blood coagulation time is delayed in parathyroid tetany. Some dogs showed only a slight delay while the blood of one in severe tetany did not clot for thirty hours. One ounce of cod liver oil or 0.4 cc. of acterol was efficacious in restoring normal coagulation time after a period of two to four hours in these animals in tetany. Animals receiving cod liver oil or acterol for twenty to forty days recovered and in this recovery the blood coagulation and serum calcium returned to normal.

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THE BIOLOGICAL VALUE OF YEAST PROTEINS FOR THE RAT

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Only a small number of the investigators have considered yeast from the viewpoint of the nutritional value of the proteins contained in it. Thomas (1909) concluded from his work that about 73.5 per cent of the yeast protein is utilized by man. Funk, Lyle and McCuskey (1916) studied the nutritional value of yeast in man, by substituting varying portions of the usual dietary proteins by yeast. They concluded that yeast cannot be recommended as a sole source of protein in the diet because a large per cent of the yeast nitrogen was of no food value, and because the ingestion of yeast was followed by a rise in the concentration of uric acid in the blood. Rubner (1916), Schattelius (1915), Wintz (1916), Winckel (1916) and Völtz (1916) found brewer's yeast to be an important food substance. Rubner's work indicates that yeast (dried) is readily digested and utilized by the animal organism. According to Völtz, yeast is one of the most digestible and valuable foodstuffs for man and dogs.

Osborne and Mendel (1919) state that on feeding brewer's yeast to rats as the sole source of protein the utilization of yeast nitrogen was from 74 to 83 per cent. Karr (1920) states, "In metabolism experiments with comparable quantities of yeast as the sole source of protein, the partition of urinary nitrogen compared favorably with what was observed after feeding the other two proteins. The utilization of yeast nitrogen was about 80 per cent."

Hawk and his co-workers (1919) found baker's yeast to be a valuable dietary product, and when used as an additionl source of protein, enhanced the growth of young rats on a milk diet. They found that rats which were growing on a bread and butter diet grew more rapidly if 5 per cent extra yeast was included in the bread. In man, they found yeast to be a highly utilized food. They state, "We have demonstrated that the nitrogen of yeast is preferred by the nutritional mechanism of certain individuals, to the nitrogen in the form of such staple foods as meat and

[1]The writers wish to express their appreciation to The Fleischmann Company and to Dr. R. E. Lee, the director of their department of applied research, for a fellowship, under tenure of which this problem was done.

wheat." One must conclude from their statements and data that they believe the substitution of a small portion of the meat nitrogen by yeast nitrogen in the diet of man not only introduces a better utilized protein but also that the yeast increases the utilization of the other proteins in the diet.

Murlin and Mattill (1923), studying the effect of baker's yeast on man and dogs, among other things concluded that in the dog the yeast was digested and utilized "to such an extent as to obscure any tendency to increase the excretion of nitrogen by the bowel." In discussing their experiments on man they state, "Since one yeast cake contained approximately 0.33 gram nitrogen and since the increase in total nitrogen of the stool was rarely equal to one-half of the amount contained in the yeast itself of the corresponding period, it is obvious that the yeast nitrogen was largely absorbed." They also state that boiled yeast is much more easily absorbed than raw yeast.

Still and Koch (1928) found that small quantities of baker's yeast (three cakes per day) did not seem to be utilized well by man, because a large portion of the added yeast nitrogen and phosphorus was eliminated in the feces.

On the basis of the amino acid content, as shown by analyses of the products of hydrolysis by Pringsheim (1913), Meisenheimer (1915) and Neuberg (1915), yeast protein may be considered suitable food for man.

It is surprising that the utilization of phosphorus, such an important dietary factor, has not been studied in previous metabolism experiments on yeast, especially since yeast is rich in phosphorus.

PLAN OF EXPERIMENTS. The experiments described in the following pages were planned to study two properties of yeast. First, it was desired to determine the value of the yeast proteins in relation to growth in rats, and, second, to determine the utilization of yeast nitrogen and phosphorus as well as the effect of yeast upon the retention of calcium in young rats.

The diets were so prepared that a certain desired per cent of the total nitrogen was in the form of yeast nitrogen. Casein was selected as the sole source of protein for the control diets because it has been shown to be a protein of high biological value and because many of the nutrition studies on rats have been done with diets containing casein. Using the control diet as a starting point, part or all of the casein nitrogen was substituted by yeast nitrogen in preparing the "yeast diets." Since it required about two grams of yeast to supply the nitrogen contained in one gram of casein, starch was diminished accordingly in order to keep the other percentages in order. The butter, salts and added vitamin "B" were the same in all of the diets.

PREPARATION OF DIETARY COMPONENTS. One hundred pounds of Fleischmann's "starch-free" yeast were obtained and all of the yeast

fractions studied were prepared from this one lot except the nucleo protein fraction, N. P. I.

Yeast I. The one pound cakes of yeast were cut into thin slices and dried on glass plates in a warm air drier (about 40°C.). The dried product was milled to 60–80 mesh and preserved in bottles in the refrigerator.

Analysis

	per cent
N	7.6
P	1.32

Yeast II. About 350 grams of fresh yeast were crumbled into small pieces and thrown into 2000 cc. boiling acidulated water (1 cc. glacial acetic acid per 2000 cc.). The suspension was stirred until the temperature reached 100°C. when it was filtered through paper. The residue was washed with 1000 cc. of a similar hot acid solution and finally with 500 cc. boiling water. The residue was dried and milled to 60 to 80 mesh and preserved in the refrigerator.

Analysis

	per cent
N	7.72
P	0.9

Vitamin "B" concentrate. The combined filtrates from the preparation of "yeast II" were evaporated to a small volume and carried through essentially the process of Osborne and Wakeman (1919). A product was obtained after two reprecipitations by alcohol which was entirely soluble in water and which contained a high concentration of the growth stimulating substance, a diet containing 0.4 per cent being adequate to insure maximum growth of young rats.

Nucleo protein N. P. I. One pound of fresh yeast was broken into small pieces and stirred into 2000 cc. of a 2 per cent solution of sodium hydroxide. The suspension was stirred vigorously until a smooth suspension was obtained. After standing for fifteen minutes at room temperature, the suspension was separated by means of a Sharples Super Centrifuge. The fluid portion coming from the centrifuge was conducted directly into a jar containing approximately five volumes of 95 per cent alcohol and slightly more hydrochloric acid than necessary to neutralize the alkali. The nucleo protein with some other substances formed a precipitate which rapidly settled out so that separation by decantation was possible. The product was finally filtered from the small amount of alcohol and dried. The yield was about 16 grams per pound of fresh yeast.

Analysis

	per cent
N	6.5
P	2.9

While the product was known to be crude and to contain other substances such as carbohydrate, protein, salts, etc., it was considered advisable to use it as thus obtained rather than to submit it to the further action of reagents.

Preparation of casein. One kilogram of crude casein was suspended in about 15 liters of cold distilled water and enough 10 per cent sodium hydroxide added to almost cause complete solution. The whole was rapidly filtered through muslin and precipitated by acetic acid. The product was washed several times with 0.1 per cent acetic acid and finally with distilled water. After dehydration with alcohol and ether the product was dried in a current of air and then milled to 60 to 80 mesh.

Analysis

	per cent
N	12.2
P	0.77

"Arlco" starch was used in the diets. The standard inorganic salt mixture prepared by the Harris Laboratories was used. Creamery butter was the source of fat in the diets.

The parts of the diets were accurately weighed or measured and after thoroughly mixing by hand were run repeatedly through a large power meat grinder. This was to insure an even distribution of the diet. The diets were made in such quantities as would last for not over one week. Glass jars were used for preserving the diets in the refrigerator.

CAGES. Many types of metabolism cages have been described for use with rats. Several types were tried and were not found entirely satisfactory for collecting samples of feces and urine for separate analyses. By combining the best features of several forms and making a modification in the separating device a very satisfactory arrangement was developed. This is shown in figure 1.

In order to obtain the best results with the metabolism cages certain precautions were observed. 1. The absorbing papers were thoroughly washed before use to remove any soluble constituents which might affect the analysis later. 2. The feces were collected from the false bottom several times a day, and preserved in stoppered bottles until ready for analysis. 3. The absorbing papers were removed each forty-eight hours and the extract thereof (five times with hot 5 per cent HCl) was placed together with the washings from the false bottom, true bottom and the funnel, in a 1 liter flask from which aliquot samples were taken for analysis. 4. Standard macro methods were used for the analysis, namely, the macro Kjeldahl for nitrogen, Neumann-Pemberton method for total phosphorus and the permanganate titration of calcium oxalate. 5. Food was changed daily.

In the studies on growth, large metal cages were used which were fitted

with false bottoms. The cages were fitted with very satisfactory food containers which prevented scattering and loss of food.

Black and white rats were used in all of the experiments reported in this paper and were raised in this laboratory from healthy and vigorous parents. A group of control animals was carried in parallel with each experiment.

EXPERIMENTS ON GROWTH. A number of experiments were carried out in which the growth of rats was observed while consuming the casein and yeast containing diets ad libitum. In all of these experiments the animals on the casein diet grew at a much more favorable rate than those on the yeast diets. However, since the animals usually ate more plentifully of the

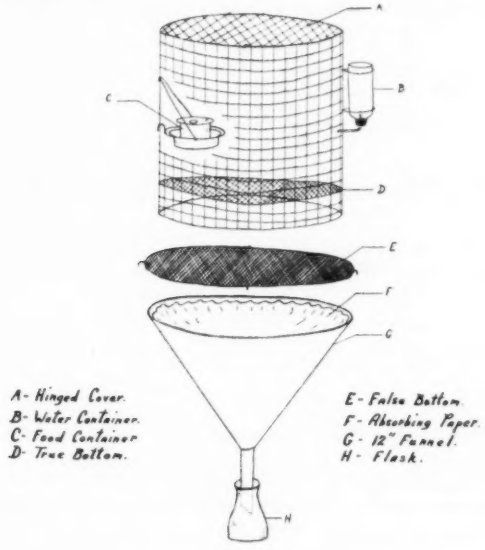

Fig. 1

casein diet the results obtained were not considered to be quantitatively comparable. For this reason the data from these experiments will be omitted and other experiments were arranged in which the food intake was caused to be similar for all diets.

Experiment I. A quantitative study of the growth promoting properties of diets in which casein and yeast preparations furnish the protein, singly or in combination.

In the control diet casein furnished one hundred per cent of the nitrogen. Other diets were made in which yeast I and yeast II furnished fifty and one hundred per cent of the nitrogen respectively. In designating the diets the first letter or letters indicate the protein source (C = casein and

Y = yeast), the Roman numeral identifies the yeast preparation used and the last figure gives what per cent of the total nitrogen is from the yeast preparation.

Diet C

	per cent	per cent
Casein	20	
Starch	60	N = 2.7
Butter	15	P = 0.78
Salts	4	Ca = 0.55
"B"	1	

Diet Y-I-100

	per cent	per cent
Yeast I	38	
Starch	42	N = 2.8
Butter	15	P = 1.2
Salts	4	Ca = 0.54
"B"	1	

Diet Y-II-100

	per cent	per cent
Yeast II	38	
Starch	42	N = 2.7
Butter	15	P = 1.1
Salts	4	Ca = 0.52
"B"	1	

Diet CY-I-50

Equal parts of diets C and Y-I-100 were mixed.

Diet CY-II-50

Equal parts of diets C and Y-II-100 were mixed.

Thirty young male rats were distributed equally among six cages and caused to consume similar quantities of food per gram of body weight per day in the following manner. All rats were given their food each day in the same amount per gram of body weight as was consumed on the previous day by a particular group of rats (the group which ate most sparingly). This system was carried on throughout the experiment. The group eating most sparingly was given the food ad libitum.

Table 1 shows the results of the experiment. Figure 2 shows the comparative growth promoting qualities of the diets when fed at similar levels. This figure is a composite made from several experiments carried out in the manner just described.

Experiment II. The same diets were used in this experiment as in the experiment just described. In this experiment six young male rats were placed in each cage. The food intake was controlled in the manner described in experiment I. The animals in the various cages were allowed to consume a given diet for a number of days, then changed to one of the

other diets. By changing the diets in this manner it was hoped to be able to confirm the observations made in experiment I in several groups of rats. Figure 3 clearly shows the manner in which the experiment was carried out, as well as the growth observed.

Examination of the data of these experiments indicates that the animals grew at a uniform rate for each diet. In every instance they grew better on diet C than on any of the "Y" diets; also diet Y-II-100 was almost equal to diet Y-I-100 and diet CY-I-50 to diet CY-II-50 in promoting growth.

It will be noted from table 1 that in cage I the animals were limited to considerably less food than those on the yeast diets, yet these animals

TABLE 1

Showing the growth of animals on the various diets under conditions of similar food intake

CAGE	DIET	NUMBER OF DAYS	AVERAGE FOOD EATEN PER GRAM OF RAT PER DAY*	WEIGHT (1)	WEIGHT (2)	GAIN IN WEIGHT	GAIN PER DAY
1	C	15	0.119	43.5	93.0	49.5	3.3
2	C	15	0.145	44.3	110.0	65.7	4.38
3	Y-I-100	15	0.144	35.6	59.4	23.8	1.59
4	CY-I-50	15	0,137	35.8	79.6	43.8	2.92
5	Y-II-100	15	0.144	42.2	56.2	22.0	1.46
6	CY-II-50	15	0.136	35.8	82.8	47.0	3.13

* These average figures were obtained in the following manner: The average weight of the rats in a cage was determined at the end of each one-day period, this weight was divided into the average food consumption per rat per day for the cage during the period.

grew much better than those on either of the Y-100 diets and slightly better than those on the Y-50 diets.

These experiments show that the casein diet is superior to the yeast diets in promoting growth in young rats. The cause of this difference is not apparent. It was thought that the difference in the mineral ratios of the diets might have been one important factor. This was investigated by two groups of experiments.

Experiment III. Diet C and Y-I-100 were so modified by the addition of calcium lactate and a neutral sodium phosphate mixture that they were of similar calcium and phosphorus content, the Ca:P ratio being 1.92. The animals were caused to consume similar quantities of food as described before.

In this experiment the animals on the modified diet C gained 61 grams

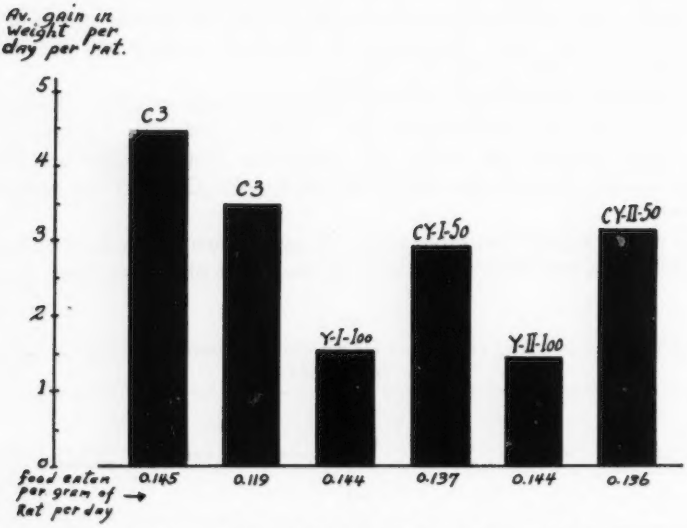

Fig. 2. A composite figure showing the growth of rats consuming similar quantities of food per unit of body weight.

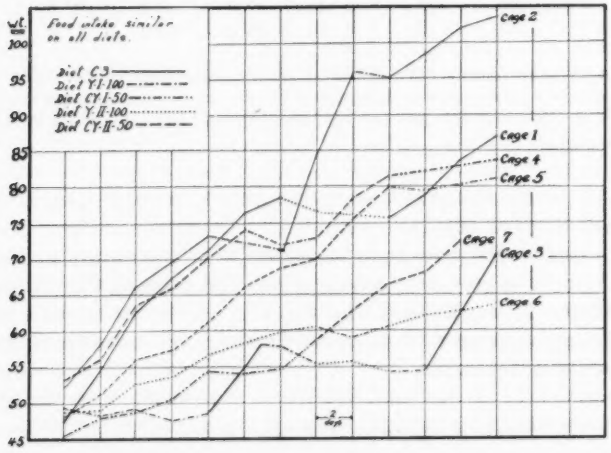

Fig. 3

in 28 days or a gain of one gram for each 2.4 grams of food eaten, while those on the modified Y-I-100 diet gained only 24 grams in the same period, their gain being one gram for each 5.1 grams of food eaten.

Although the above experiment seemed to show that the mineral ratio was not an important factor influencing the growth of the animals, another experiment was carried out to clarify the point.

Experiment IV. Diets C and Y-I-100 were prepared without the four per cent of salts. To diet C were added the ash from the amount of yeast in diet Y-I-100, 2 per cent NaCl and 0.5 per cent CaO. To diet Y-I-100 (without salts) were added the ash from the amount of casein in diet C, 2 per cent NaCl and 0.5 per cent CaO. These diets are numbered re-

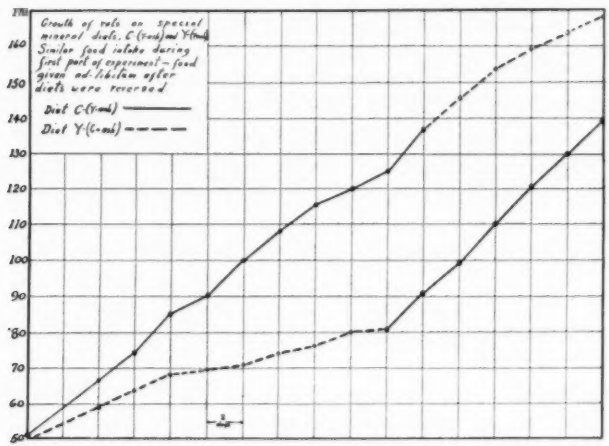

Fig. 4

spectively C-(Y ash) and Y-(C ash). The ash of yeast is known to be a satisfactory source of minerals with the exception of calcium and NaCl, hence the addition of the calcium and NaCl. These diets then were of of the same nitrogen and mineral content, the only difference being in the source of the nitrogen (protein) and amount of starch. Growth experiments, at similar levels of food intake, should reflect the comparative values of the protein in the diets and at the same time clarify the question of the mineral content referred to.

Figures 4 and 5 show the growth of the animals on these diets under similar conditions of food intake.

Discussion of growth experiments. All of these experiments have been made under similar conditions of food intake per gram of rat per day. Since the diets used were of similar composition except for the

source of protein (and of starch, since a larger amount of yeast was used than of casein to supply the desired amount of nitrogen), it seems obvious that the yeast proteins calculated on the basis of the nitrogen content are not as satisfactory as casein for growth purposes in the rat. A study of figure 5 shows that rats which were consuming diets which may be said to be identical except for the source of protein and starch content, grew at a much better rate on the casein diet than on the diet containing yeast. The rats on the casein diet gained approximately 2 grams per day more than those on the yeast diet. By reversing the diets in this experiment it was possible to observe the growth curves of the rats to cross twice, the curve of the control rats rising above the curve of the "yeast" rats in each

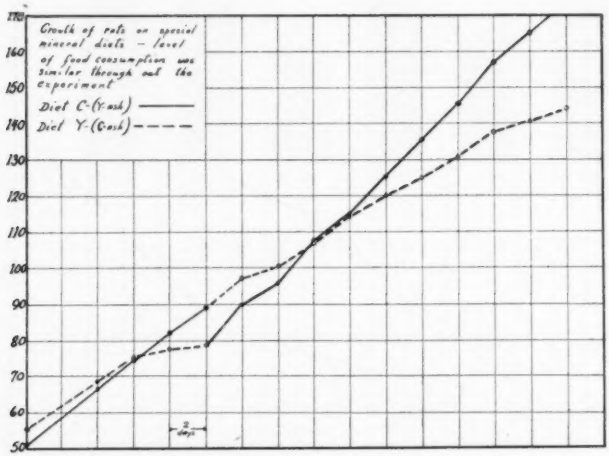

Fig. 5

instance. We believe this figure (5) portrays accurately the comparative nutritive values of the two diets.

Although analyses do not indicate an amino acid deficiency in the yeast protein it was thought that possibly some of the "essential" amino acids were not available for metabolism. Although this point has not been thoroughly investigated we added histidine and tryptophane to the Y-100 diets without any observable improvement. It appears then that the unfavorable growth on the yeast diets is not due to an amino acid deficiency.

A few of the young rats developed a severe diarrhea during the period on the yeast diets. This was considered to be a manifestation of the sensitivity of the individuals' intestines. This view is supported by the recent work of Thorup and Carlson (1928) who found that 25 per cent of

yeast added to a normal diet did not increase the intestinal rate of rats. Data from rats showing the diarrhea were not included in our tables and figures.

From these experiments one must conclude that yeast as such is not as satisfactory a source of protein in the diet of the rat as casein, and that on substituting part or all of the casein in the diet by yeast one decreases the value of the diet in proportion to the amount of casein substituted.

These experiments suggest that the yeast product I is slightly better than yeast product II. If this suggestion is true the difference may be due to the extraction of a valuable food substance in preparing yeast II, or to detrimental changes induced by heat coagulation.

These findings are contrary to those reported by Hawk, Smith and Bergeim (1921) who found that yeast added to a milk diet increased the rate of growth of young rats. On that basis they suggested yeast as an adjunct for use in infant feeding. Our results as well as those of Daniels (1922) contraindicate the use of yeast for such purposes. One would hardly expect growing rats to make maximum growth on a milk diet and it is to be expected that any increase in the quantity of protein consumed (even though only partly utilizable) would be followed by an increase in the rate of growth. However, it is impossible to evaluate their experiments since they do not give any data concerning the quantity of food eaten nor the amount of yeast added.

The same authors (1921) reported better growth in rats on a bread, butter and salts diet by incorporating five per cent extra yeast (dry powder) in the bread. Possibly their control diet was low in vitamin B, that the addition of yeast corrected this and thus permitted better food consumption and growth. However, since they do not give any data concerning the food consumption one cannot evaluate the work.

Nelson, Heller and Fulmer (1923) found yeast to supply good proteins. None of their growth curves indicate growth on the yeast diets of similar magnitude as was obtained on the casein diets. We interpret this as meaning that the growth promoting qualities of the yeast protein are inferior to casein. Our work confirms them in that our rats did not grow normally on yeast as the sole source of protein. We have not confirmed them in their statement that yeast proteins are good proteins in that we were unable to obtain good growth of rats on diets containing yeast in such amounts that they were equivalent in nitrogen content to diets containing from 18 to 20 per cent of casein. Since they do not give the quantities of food eaten one must look upon their experiments as suggestive rather than conclusive.

METABOLISM EXPERIMENTS. The plan of the experiments was to study further the growth of somewhat more mature rats on the C and Y diets simultaneously with the collection and analysis of the excreta. It was

hoped that the data would not only throw further light upon the growth phenomena but would also indicate the relative utilization of the various diets. The diets used in these experiments were similar to those used in the growth studies except that the nitrogen in the yeast diets was increased. We have, however, used the same numbers for identifying the diets because the same preparations were used therein.

The new diets, CX, N P I, and Y-II-100-X were prepared to study the nucleo-protein fraction of yeast and the soluble proteins of yeast. The Y diet followed by the letter H indicates that the yeast was heated to 100 degrees in order to destroy the enzymes.

Diet C

	per cent		per cent
Casein	20	Total N	= 2.50
Starch	60	Soluble N*	= 0.34
Butter	15	Total P	= 0.70
Salts	4	Soluble P*	= 0.55
"B"	1	Ca	= 0.58

* The soluble nitrogen and phosphorus in the diets were determined as follows: 10 grams of the diet were extracted five times with 5 per cent HCl in the cold for ten minutes each time. The combined extracts were made to volume and aliquots taken for analysis.

Diet Y-I-100

	per cent		per cent
Yeast I	40	Total N	= 3.40
Starch	40	Soluble N	= 0.77
Butter	15	Total P	= 1.08
Salts	4	Soluble P	= 0.62
"B"	1	Ca	= 0.47

Diet CY-I-50

	per cent		per cent
Yeast I	20	Total N	= 2.90
Casein	10	Soluble N	= 0.56
Starch	50	Total P	= 0.88
Butter	15	Soluble P	= 0.58
Salts	4	Ca	= 0.53
"B"	1		

Diet Y-II-100

	per cent		per cent
Yeast II	40	Total N	= 3.31
Starch	40	Soluble N	= 0.35
Butter	15	Total P	= 0.90
Salts	4	Soluble P	= 0.46
"B"	1		

Diet CY-II-50

	per cent		per cent
Yeast II	20	Total N	= 2.95
Casein	10	Soluble N	= 0.35
Starch	50	Total P	= 0.80
Butter	15	Soluble P	= 0.51
Salts	4	Ca	= 0.49

Diet N P I

One hundred grams of diet C were mixed with 4.6 grams of the nucleoprotein N P I (the amount obtained from the quantity of yeast used in preparing diet Y-I-100).

	per cent
Total N	= 2.77
Soluble N	= 0.51
Total P	= 0.80
Soluble P	= 0.62
Ca	= 0.63

Diet CX

Forty grams of yeast I were extracted with 250 cc. of boiling water and filtered, the residue was washed twice with a similar amount of boiling water. The combined filtrates were evaporated under reduced pressure to a thick syrup. One hundred grams of diet C were mixed with this syrup.

	per cent
Total N	= 2.73
Soluble N	= 0.66
Total P	= 0.80
Soluble P	= 0.66
Ca	= 0.49

Diet Y-II-100-X

One hundred grams of diet Y-II-100 were mixed with an extract prepared in the same manner as given under diet CX.

	per cent
Total N	= 3.50
Soluble N	= 0.79
Total P	= 1.07
Soluble P	= 0.62
Ca	= 0.40

Twelve vigorous male rats (100–116 grams) were selected and placed in separate metabolism cages (see fig. 1). Two animals were treated alike in the experiment, one being a check upon the other. The figures are averages for the two animals. In every case the analytical results obtained compared very closely for the two animals. After a preliminary period of two days on the diet to be studied to insure no subsequent contamination of the excreta by the previous diet, the experiment was begun. Four days constituted one analytical period. Samples were collected and preserved as described earlier in the paper.

Due to the fact that it was very difficult to sample the feces the following procedure was adopted. The air dried feces were ground in a mortar to obtain a finely divided state and thoroughly extracted with warm ten per cent hydrochloric acid. The filtrate contained the calcium, soluble

TABLE 2

Showing analytical data from cages 1 and 1a (averaged)

PERIOD	DIET	AVERAGE WEIGHT	FOOD				URINE						FECES										BALANCE			WEIGHT OF DRY FECES PER RAT PER DAY
			Eaten per gram of rat per day	N	P	Ca	N		P		Ca		Nitrogen				Phosphorus				Calcium		N	P	Ca	
							Total	Per cent.*	Total	Per cent.*	Total	Per cent.*	Total	Soluble	Insoluble	Per cent.*	Total	Soluble	Insoluble	Per cent.*	Total	Per cent.*				
1	C	120½	0.108	336																						
2	C	133	0.096	331	89.7	74.7	164	49.5	31.2	34.7	6.02	8.05	25.9	12.6	13.3	7.8	27.07	26.7	0.37	30.8	39.3	52.6	+141.1	+31.43	+29.38	0.7
3	C	141½	0.085	313	84.8	70.6	173	55.2	33.7	39.7	6.93	9.81	29.2	15.4	13.8	9.3	27.84	27.5	0.34	32.8	40.2	56.9	+110.8	+23.2	+20.7	0.5
4	NPI	149½	0.074	312	90.6	70.7	201	64.4	41.9	46.2	6.95	9.82	30.0	12.5	17.5	9.9	29.96	29.4	0.56	33	42.2	59.6	+72	+18.7	+21.6	0.6
5	NPI	156½	0.067	290	84.2	65.7	188	64.8	43.4	51.5	6.37	9.69	30.0	12.0	18.0	10.3	26.36	25.9	0.46	31.3	38.7	58.8	+72	+14.4	+19.6	0.6
6	C	161	0.058†	243	65.9	54.9	175½	76.3	34.8	52.6	4.25	7.74	25.4	12.6	12.8	14.5	23.2	22.8	0.38	35.1	36.7	66.2	+42.1	+7.9	+13.9	0.35

TABLE 3

Showing analytical data from cages 2 and 2a (averaged)

1	Y-I-100	109	0.0917	339																						
2	Y-I-100	111	0.0846	319	101.2	43.9	150	47	40.6	40.1	4.41	10.0	106	38.5	67.5	33.2	42.4	38.4	4.0	41.8	25	56.9	+63	+18.2	+14.49	1.3
3	Y-I-100	115	0.0843	329	104.5	45.3	185	56.2	48.5	46.4	6.25	13.7	113.1	38.5	74.6	34.3	51.2	47	4.2	48.9	34	75	+30.1	+4.8	+5.05	1.4
4	C	130½	0.0961	323	87.6	73	184	56.9	30.4	34.7	6.35	8.7	38.5	12.7	25.8	11.9	32.5	30.7	1.8	37.1	44.3	60.6	+100.5	+24.7	+22.35	0.56
5	C	149	0.0839	323	87.6	73	148	45.8	34.6	39.4	6.06	8.2	22.2	9.0	13.2	6.9	24.4	23.4	1.0	27.8	38	52	+152.8	+27.6	+28.9	0.26
6	CX	167½	0.0808	368	108	65.5	197.4	53.6	55	50.9	6.1	9.3	27.7	12.3	15.4	7.5	27.1	25.9	1.2	25	39.7	60.6	+142.9	+25.9	+19.7	0.45

TABLE 4

Showing analytical data from cages 3 and 3a (averaged)

1	Y-I-100-H	108½	0.094																							
2	Y-I-100-H	104½	0.088	312	99.08	43	157	50.3	42.6	42.9	4.67	10.8	116	48	68	37.1	41.14	38.1	3.04	40.5	32.4	75.3	+39	+15.25	+5.92	1.5
3	Y-I-100-H	109	0.0954	353	112	48.6	179	50.4	48.4	43.2	5.43	11.1	131.1	46.8	84.3	37.1	44.4	39.6	4.8	39.6	30.6	62.9	+43	+19.2	+12.57	1.75
4	C	124	0.10	323	87.6	73	201	62.2	32.0	36.6	6.48	8.87	29.5	8.3	21.2	9.1	21.3	19.9	1.4	24.3	40	54.7	+92.5	+34.3	+26.5	0.56
5	CY-II-50	138½	0.09	368	100.1	61.25	184	50	46.0	45.9	5.4	8.98	103.3	29.3	74	28.0	38.47	35.1	3.37	38.3	45.7	74.6	+80.7	+15.53	+10.1	1.19
6	CY-II-50	143	0.065	277	75.3	46.06	148.8	53.7	37.8	50.2	5.55	12	65.2	13.2	52	23.5	25.23	22.6	2.63	33.5	25.4	55.2	+63	+12.27	+15.1	1.0

* Expressed as per cent of the food value.

† Limited.

† Limited.

TABLE 5

Showing analytical data from cages 4 and 4a (averaged)

PERIOD	DIET	AVERAGE WEIGHT	FOOD				URINE						FECES										BALANCE			WEIGHT OF DRY FECES PER RAT PER DAY
			Eaten per gram of rat per day				N		P		Ca		Nitrogen				Phosphorus				Calcium					
				N	P	Ca	Total	Per cent*	Total	Per cent*	Total	Per cent*	Total	Soluble	Insoluble	Per cent*	Total	Soluble	Insoluble	Per cent*	Total	Per cent*	N	P	Ca	
1	Y-II-100	112½	0·106																							
2	Y-II-100	114½	0·099	377	102·8	45·1	153	40·58	48·2	46·8	5·82	12·9	183	30·2	153·8	48·5	44·7	36·3	8·4	43·4	26·3	58·3	+41	+9·9	+13·0	1·65
3	Y-II-100	115½	0·09	344	93·8	41·2	172	50	45·2	48·1	6·28	15·2	138	39·3	98·7	40·1	36·25	32·2	4·05	38·6	34·6	83·9	+34	+12·3	+0·4	1·5
4	C	129	0·096	323	87·6	73	209	64·7	34·5	39·3	7·75	10·6	41·2	12·9	28·3	12·7	33·75	31·9	1·85	38·5	46·25	63	+72·8	+15·35	+19	0·63
5																										
6																										

TABLE 6

Showing analytical data from cages 5 and 5a (averaged)

1	Y-II-100-X	113	0·105																							
2	Y-II-100-X	115	0·100	403	122·5	46·23	172	42·6	51·3	41·8	4·45	9·8	155·7	35·7	121	38·6	42·33	36·9	5·43	34·5	24·5	52·9	+75	+28·8	+17·2	1·7
3	Y-II-100-X	121½	0·087	371	112·9	42·6	197	53·1	59·4	52·6	5·48	12·8	120·7	27·7	93	32·5	42·37	38·0	4·37	37·5	29·9	70·1	+53·3	+11·1	+7·22	1·45
4	C	134	0·0932	323	87·6	73	201	62·2	33·4	38·09	6·75	9·24	43	14·0	29	13·3	31·36	29·0	2·36	35·8	46	63	+79	+22·8	+20·2	0·62
5	Y-I-100	136	0·0654	302	95·9	41·56	168	55·6	44·3	46·2	4·25	10·2	93	26·5	63·5	29·8	43·48	38·0	5·48	45·3	32	76·9	+44	+8·12	+5·3	1·2
6	Y-II-100	142½	0·080	380	103·7	45·54	180	47·3	45	43·3	6·21	13·6	159·6	40·6	119	42	42·72	39·9	2·82	41·1	31·5	69·1	+40·4	+15·9	+7·8	2·1

TABLE 7

Showing analytical data from cages 6 and 6a (averaged)

1	CY-I-50	115	0·104																							
2	CY-I-50	126	0·0952	348	105·6	63	158	45·4	39·1	37·0	5·55	9·0	93·8	30	69·8	26	40·29	37·8	2·49	38·1	34·5	54·7	+99·2	+26·2	+22·9	1·0
3	CY-I-50	131	0·08	304	89·4	55·1	165	54·2	38·7	43·3	6·93	11·0	75·2	24·7	50·5	24·7	36·8	34·5	2·3	41·1	35	55·5	+63·8	+13·1	+13·1	0·94
4	C	141½	0·083	305	82·7	68·9	165	54·1	28·8	34·8	7·06	10·2	30	9·0	21	9·83	22·86	21·7	1·16	27·6	32	46·4	+110	+31	+29·8	0·43
5	Y-II-100	144	0·0715	341	92·9	40·8	174	51	51	54·8	6·27	15·3	104·6	28·0	76·6	30·6	39·09	36·6	2·49	42	38	93·1	+62·4	+2·8	−4·19	1·5
6	Y-I-100	142	0·058	272	86·2	37·4	171	62·7	51·2	61·7	6·25	16·7	104·9	31·3	73·6	38·5	38·93	35·2	3·73	44	29·9	79·9	−3·9	−5·9	+1·2	1·3

* Expressed as per cent of the food value.

nitrogen and phosphorus. The filtrate was made up to volume and aliquot samples taken for total nitrogen, total phosphorus and calcium analysis. The entire insoluble residue was digested with sulphuric acid and aliquot parts analyzed for nitrogen and phosphorus. From the figures thus obtained the content of the feces was calculated.

One group (two cages) constituted the control group, the other five groups were used to study the Y diets. The first period was used for the

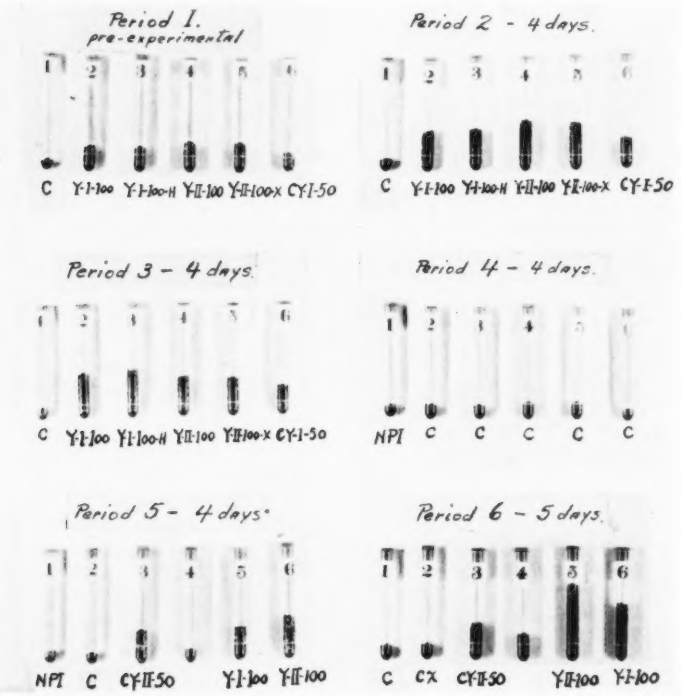
Period I.
pre-experimental
Period 2 - 4 days.
Period 3 - 4 days.
Period 4 - 4 days.
Period 5 - 4 days.
Period 6 - 5 days.
C Y-I-100 Y-I-100-H Y-II-100 Y-II-100-X CY-I-50
NPI C CY-II-50 Y-I-100 Y-II-100

Fig. 6. Showing the mass of the feces from the metabolism experiments. Numbers on tubes represent cage numbers.

preliminary period referred to above, the second and third periods for the study of the Y diets. During the fourth period all animals were placed on the control diet and during the fifth and sixth periods the Y diets were again studied.

Tables 2 to 7 inclusive show the analytical data collected during these experiments.

It is shown in tables 2 to 7 that in every case when an animal was given

diet C there followed a marked decrease in the weight of the feces as compared with other diets. A corresponding decrease in the fecal nitrogen and phosphorus was also noted. However, there is no direct relation between the weight of the feces and the analytical findings. The lowest nitrogen figures (in per cent) for the feces were obtained in every case during the period on the control diet. Also the highest urinary figures (in per cent) for nitrogen were obtained during the control periods. The higher nitrogen balance figures on the control diet suggest a more complete digestion and absorption of the casein.

If one expresses the insoluble fecal nitrogen as per cent of the insoluble food nitrogen one finds for the animals on diets C, CX and N P I from five to eight per cent; on diets CY-I-50 and CY-II-50 from 22 to 25 per cent and for diets Y-I-100 and Y-II-100 from 30 to 35 per cent. Since the nitrogen balances on these diets are in inverse order to the fecal nitrogen figures, one must conclude that the lower balances on the Y diets are in a large measure due to the losses through the feces. That these losses are not caused by an increased intestinal rate is concluded from the work of Thorup and Carlson (1928).

Figure 6 shows the mass of the feces collected during this experiment.

The fact that apparently considerable nitrogen may be absorbed from the yeast diets without satisfactory growth or nitrogen balances, indicates that the yeast nitrogen which is absorbed is not in a readily utilizable form.

A higher phosphorus balance was observed on the control diet than on any of the Y diets. This is rather surprising since yeast is a rich source of phosphorus and since during the control periods the phosphorus intake was lower than during the periods on the yeast diets. This observed higher phosphorus balance on the control diet may have been the result of many factors, among which are:

1. The casein phosphorus may have been in a better form for utilization than the yeast phosphorus.

2. The differences in the Ca:P ratios may have been an influencing factor.

Sufficient information is not available to permit one to draw conclusions as to the best forms of phosphorus for nutritional purposes nor to correctly evaluate the value of the protein-phosphorus except in terms of the net daily balance. We do not believe the Ca:P ratio to be important in this case because, as will be shown later, the ratio was satisfactory to permit satisfactory calcium balances.

Tables 2 to 7 show that on all diets there was only a small per cent of insoluble fecal phosphorus as compared with the per cent of insoluble food phosphorus. This is evidence that the phosphorus of the food was hydrolyzed into a soluble form and may be interpreted as meaning that the yeast phosphorus was available for absorption to almost the same degree

as the casein phosphorus. On the other hand there was an increase of phosphorus in both the urine and feces on the yeast diets as compared with the control diet. This indicates that the yeast phosphorus which was absorbed was not well utilized and also not as well absorbed as the casein phosphorus. Table 2 shows that the nucleo-protein prepared from yeast (diet N P I) is not a satisfactory source of either nitrogen or phosphorus since slightly more than the nitrogen and phosphorus added in this form were rapidly excreted in the urine and feces, most of the loss being through the urine. In view of these facts it seems highly probable that the low phosphorus balance on the yeast diets may be explained in part by an incomplete digestion of the yeast protein and in part by the fact that the nucleo-protein is not utilized well. This accounts for the loss of phosphorus in the urine and feces.

The excretion of calcium in the urine was quite similar and constant on all diets, however, the fecal calcium in all cases was higher during the periods on the yeast diets as compared with the periods on the control diet. The calcium balance was satisfactory during the control periods but was inferior during the periods on the yeast diets. While the Ca:P ratio was different for each of the diets, in all cases this ratio was such as would have permitted satisfactory calcium and phosphorus balances providing we assume the forms of calcium and phosphorus were equally available. Comparing the intake of calcium and phosphorus of our control groups with the similar intakes of Haag and Palmer (1928) it will be seen that similar balances were obtained. However, their animals receiving diets containing calcium and phosphorus in quantities similar to our yeast diets retained more of the calcium and phosphorus than our animals when on the yeast diets.

That the low phosphorus and calcium balances on the yeast diets are not entirely due to the Ca:P ratio but rather to some other factors is made quite obvious from a study of table 8. From this table one may observe that during the period on diet CX the Ca:P ratio was 1.64. During this period the calcium and phosphorus balances were quite satisfactory. During the period on diet CY-II-50 the Ca:P ratio was 1.63; however, only half as much calcium and phosphorus was retained. Other similar comparisons of the casein diets with the yeast diets show that the low mineral balances were not entirely due to a faulty mineral ratio.

It seems probable that the mineral ratio is not the only factor which is responsible for the lower calcium and phosphorus balances on the yeast diets. No doubt the large amount of phosphorus excreted in the feces of the animals on the yeast diets was one important factor in the calcium losses.

Table 8 shows the nitrogen, phosphorus and calcium contained in the

food, the daily balance and the balance in per cent of the daily intake. This table contains the average data from all experiments (from tables 2 to 7). Since all of the figures of various experiments are averages for a given diet in this table, it cannot be used for determining the actual values of the diets due to overlapping in the collections of the samples for analysis.

TABLE 8

Showing the intake, balance and per cent balance of N, P and Ca on all diets

The data in this table represent the averages for each diet, taken from tables 2–7.

DIET	NITROGEN			PHOSPHORUS			CALCIUM		
	In food	Balance	Per cent	In food	Balance	Per cent	In food	Balance	Per cent
C	321	107.4	33.0	86.9	26.3	31.0	72.4	24.6	33.7
CX	368	142.9	38.8	108.0	25.9	23.0	65.5	19.7	30.0
NPI	301	72.0	23.5	87.4	16.6	18.9	68.2	20.6	30.2
Y-I-100	306	33.3	10.5	96.9	6.29	6.0	42.0	6.5	15.0
Y-I-100-H	332	41.0	12.4	106.6	17.2	16.2	45.8	9.24	19.5
CY-I-50	326	81.5	24.7	97.5	19.6	19.7	59.0	18.0	30.1
Y-II-100	361	44.5	12.4	98.3	10.3	10.3	43.2	4.28	9.2
Y-II-100-X	387	64.1	16.5	117.7	20.0	16.6	44.4	12.3	27.1
CY-II-50	322	71.5	22.3	87.7	13.9	18.8	63.6	12.6	24.6

TABLE 9

Values obtained from those experiments which were controlled by a preliminary period on the same diet

DIET	FOOD N	URINE N		FECAL N		BALANCE		ENDOGENOUS FECAL N	ENDOGENOUS URINARY N
		Total	Per cent of food N	Total	Per cent of food N	In milligrams	Per cent of food N		
C*	322	161.6	50.1	25.7	8.7	135.5	36.6	25.0	20.5
Y-I-100	324	167.0	51.6	109.5	35.7	46.5	14.4	19.0	15.4
CY-I-50	360	162.5	45.3	160.0	44.3	37.5	10.3	22.8	16.1
Y-II-100	326	161.0	49.8	83.0	25.4	81.5	24.7	23.4	18.2
CY-II-50	322	166.4	51.8	84.2	25.8	71.8	22.3	21.8	20.0
Y-I-100-H	332	168.0	50.4	123.5	37.1	41.0	12.4	19.6	14.9
Y-II-100-X	387	184.5	47.8	138.7	35.5	64.1	16.5	32.0	16.8

* Calculated from experiments in which there had been a preliminary period on the same diet.

DETERMINATION OF COEFFICIENT OF DIGESTIBILITY AND BIOLOGICAL VALUES OF THE PROTEIN IN THE DIETS. The endogenous nitrogen metabolism of the rat may be determined by the analysis of the excreta during a period on a nitrogen free diet following essentially the procedure of Mitchell (1926). Under these conditions litter mates of the rats used

in these experiments, weighing from 100 to 120 grams, excreted from 180 to 220 mgm. of endogenous fecal nitrogen per 100 grams of food consumed. The average figure (200 mgm.) is about that which was found by Mitchell (1926) for similar rats weighing 100 to 140 grams. He found 190 milligrams of endogenous fecal nitrogen per 100 grams of food consumed. He found the endogenous urinary nitrogen of these rats to be 14 to 20 mgm. per 100 grams of body weight. Our figures for six rats are from 12 to 20 mgm. per 100 grams of body weight, the average being 14 mgm.

In calculating the coefficient of digestibility and the biological values of the protein in the diets, 14 mgm. of endogenous urinary nitrogen per 100 grams of body weight and 2 mgm. of endogenous fecal nitrogen per gram of food consumed have been used in correcting the urinary and fecal nitrogen found on experimental diets.

TABLE 10

Metabolism data corrected for endogenous nitrogen excretion; coefficient of digestibility and biological values

DIET	FOOD N	FECAL N		URINARY N			COEFFICIENT OF DIGESTIBILITY		BIOLOGICAL VALUE		
		Corrected	Per cent of food N	Corrected	Per cent of food N	Per cent of absorbed N	Apparent	Corrected	Apparent	Corrected	Per cent value of C
C	322	0.7	0.2	141.1	43.8	43.9	91.7	99.3	41.9	55.9	100.0
Y-I-100	324	90.5	27.9	151.6	46.7	64.9	66.2	72.06	14.3	25.2	45.1
CY-I-50	326	60.6	19.8	142.8	43.7	53.8	74.5	81.4	28.0	37.6	67.2
Y-II-100	360	137.2	38.1	146.4	40.1	65.7	55.5	61.8	10.4	21.2	37.9
CY-II-50	322	62.4	19.3	146.4	45.4	56.3	73.8	80.6	22.2	37.9	67.8
Y-I-100-H	332	103.9	31.2	153.1	46.1	67.1	65.8	68.7	12.3	22.5	40.2
Y-II-100-X	387	116.7	30.1	167.7	43.3	62.0	68.1	70.0	16.5	26.5	47.4

Table 9 shows the averages for nitrogen intake, excretion and balance taken from only those experiments in which the feeding periods were controlled by a preliminary period on the same diet. This precaution is necessary in assembling data to be used in calculating the various coefficients and other values.

Table 10 shows such corrected values for the nitrogen metabolism of the rats, the apparent and corrected digestibility of the proteins in the diets and the apparent and corrected biological values of the protein in the diets.

Apparent digestibility: $$\frac{\text{Food N} - \text{Fecal N (uncorrected)}}{\text{Total Food N}}$$

$$\text{Digestibility}_{\text{(corrected)}}: \quad \frac{\text{Food N} - (\text{Fecal N} - \text{Endogenous Fecal N})}{\text{Total Food N}}$$

$$\text{Apparent biological value}: \quad \frac{\text{Food N} - (\text{Fecal N} - \text{Urine N})}{\text{Food N}}$$

$$\text{Biological value}_{\text{(corrected)}}: \quad \frac{\text{Food N} - \text{Fecal N}_{\text{(corr.)}} - \text{Urine N}_{\text{(corr.)}}}{\text{Food N}}$$

DISCUSSION. We wish to emphasize that our studies were conducted throughout in a comparative way. We purposely chose a high protein level because of previous good reproduction on this level with the control diet and because any difference in digestibility and availability of yeast nitrogen should be detected more easily on such a level. The rats in these experiments were consuming comparable quantities of food and especially of nitrogen. The experiments were controlled not only by a separate control group consuming the control diet but in many cases each experimental group at one time during the observations was placed on the control diet. All studies were made in duplicate and always on two or more animals. The rate of growth was confirmed in some cases by parallel observations on the nitrogen, calcium and phosphorus utilization. We have assumed all of the yeast nitrogen as protein nitrogen in our calculations. We realize that this assumption is not correct but inasmuch as a reliable value for the protein content of yeast is not available we have no better choice.

TABLE 11

Showing the per cent of the absorbed nitrogen which was retained in the body on all diets*

C	C-I-100	CY-I-50	Y-II-100	CY-II-50	Y-I-100-H	Y-II-100-X
56.1	35.1	46.2	34.3	43.7	32.9	38

*In this calculation it is assumed that the endogenous fecal and urinary nitrogen is the same on a protein diet as on a nitrogen-free diet. Making this assumption enables us to express the per cent of retained nitrogen as follows: Total food nitrogen —(corrected fecal nitrogen) = absorbed nitrogen.
Then,

$$\frac{\text{Absorbed nitrogen} - \text{corrected urinary nitrogen}}{\text{Absorbed nitrogen}} = \text{per cent of absorbed nitrogen retained.}$$

Table 10 shows that nitrogen is lost on the "Y" diets in the feces to the extent of 19 to 38 per cent. That this is due to the indigestibility of the yeast proteins is shown by the coefficients of digestibility (table 10) and by the bulk of feces as shown in figure 6.

The coefficient of digestibility of raw dried yeast (diet Y-I-100) is 72 per cent and of washed coagulated yeast (diet Y-II-100) is 61.8 per cent. This indicates that the soluble proteins of yeast are of relatively higher

nutritional value than the insoluble coagulated ones. Further evidence of this is found in the calculated biological values of these two diets and of the percentage of absorbed nitrogen which is retained by the body.

The addition of the soluble extract to diet Y-II-100 in preparing diet Y-II-100-X, increased its biological value from 21.2 per cent to 26.3 per cent. This seems to be only suggestive that the soluble yeast proteins are of a higher biological value than the coagulable ones and that heat coagulation of yeast lowers the coefficient of digestibility and biological value of the protein. This, however, is not to be emphasized because the data only suggest the point and do not prove it.

Evidence is given which indicates that the absorbed nitrogen from the "Y" diets is not as satisfactory as that from the control diet. It is not retained as well. This is shown in table 11. The soluble fraction, as is shown, causes diet Y-I-100 to be better in this respect than diet Y-II-100 and diet CY-I-50 better than CY-II-50.

Our experiments are not in harmony with the conclusions of Karr (1920) who reported yeast easily digestible and a valuable dietary protein. He reported 80 per cent utilization of yeast. Since he used brewer's yeast and experimented on dogs it is not best to make direct comparison between his work and ours. However, a careful examination of the limited data given by him actually indicates that yeast nitrogen is not utilized as well as casein or gluten nitrogen.

Osborne and Mendel (1919), in a very brief paper, report the utilization of brewer's yeast protein to be 74 to 83 per cent. This is not confirmed by our work. They do not give the figures for the nitrogen content of their diets, amounts of food eaten nor analyses of excreta. They simply state that "an estimation of the digestibility of the food tested by comparison of the feces with the intake showed a nitrogen 'utilization' in several cases of 74 to 83 per cent." In their studies they did not make comparative tests with a definite protein known to be biologically valuable.

We have not confirmed the work of Rubner (1916), Schatelius (1915), Wintz (1916), Winckel (1916) or Völtz (1916) who found brewer's yeast to be an important food substance. These workers used brewer's yeast while we used baker's yeast. This may have been one of the factors causing the difference in their conclusions and ours. It is impossible to make direct comparison between their work and ours because these workers used brewer's yeast and apparently did not control their experiments very well. Rubner experimented on a dog and fed it a diet containing 44 grams of nitrogen per day, 14 grams of which came from yeast and 30 grams from meat. Although he does not give the weight of his one animal, probably the nitrogen content of the diet was abnormally high. He studied the animal for only three days which is entirely too brief a period and he does not mention any change in weight during the

experiment. He admits observing a loss of calories in the feces, intestinal disorders and diarrhea during the ingestion of yeast. However, inasmuch as the animal absorbed a relatively large portion of the yeast nitrogen he concludes that the yeast contains valuable nutritional protein.

Schattelius (1915) studied the effect of substituting the usual dietary proteins, in part, by yeast in the diets of man. His criterion was the maintenance of body weight. Since his diets contained other proteins of high biological value and since the adult man can remain in weight equilibrium on quite small quantities of protein providing the other food substances are of sufficient quantity, it is felt that his experiments are probably of little value in determining the biological value of the yeast proteins.

CONCLUSIONS

1. Young rats will grow on diets containing yeast as the sole source of protein. However, the growth on such diets is not as satisfactory as is obtained on diets of similar composition containing casein as the source of protein. Older rats are able to maintain themselves and grow better on the yeast diets than younger ones. A few of the younger rats showed intestinal disturbances as manifested by severe diarrhea. This was especially noticed on diets containing unwashed yeast and probably can be explained in part on the basis of the higher salt concentration as compared with the diets containing washed yeast.

2. The better growth on the casein diets as compared with the yeast diets probably cannot be attributed to an amino acid deficiency of the yeast protein.

3. The coefficient of digestibility of the dried, raw yeast protein (assuming all of the nitrogen to be protein nitrogen) is 72 per cent.

4. The coefficient of digestibility of the coagulated yeast protein (assuming all of the nitrogen to be protein nitrogen) is 62 per cent.

5. As compared with casein, the biological value of the dried raw yeast proteins is 45 per cent.

6. As compared with casein, the biological value of the coagulated yeast proteins is 37.9 per cent.

7. Yeast is not as satisfactory a source of protein in the diet of the rat as is casein.

8. Yeast is not as satisfactory a source of phosphorus in the diet of the rat as are casein and neutral inorganic phosphate mixtures.

9. Diets containing yeast to the extent of supplying 50 to 100 per cent of the total nitrogen are not as favorable from the viewpoint of calcium retention as diets containing similar amounts of casein nitrogen.

10. The administration of yeast in the quantities usually recommended for therapeutic purposes is not contraindicated because the quantity of yeast protein so consumed is small as compared with the total protein consumption.

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DIET PROTEINS AND VITAMINS AS RELATED TO HEMOGLOBIN PRODUCTION IN THE RAT

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The problem of the origin of hemoglobin has occupied the minds of physiological chemists and the medical profession for many years. A review of the literature cannot but impress one with the importance generally ascribed to the diet in this connection. It is admitted that hemoglobin synthesis is normally a continuous process in both growing and fully grown animals. The building blocks for this synthesis must normally be present in the diet. Whatever may be the immediate organic precursors of hemoglobin, it is obvious from experiments with growing rats on synthetic diets that these precursors are to be found among the proteins or protein split products of the diet. Thus it would seem possible that a diet might be chosen which would be deficient in these precursors and which would produce anemia in an animal subjected to such a diet. The attempts to produce and cure such anemias have been frequent and the results have been varied. In recent times, the question of the relation of vitamin intake to hemoglobin formation has received no small amount of attention.

Geiling and Green (1921) report that diets deficient in proteins, vitamins or minerals delayed regeneration of blood in rats made anemic by bleeding. Similarly, Jencks (1922) found that protein produced a more rapid regeneration of blood than either fat or carbohydrate when fed alone. She also reports that blood regeneration was faster upon diets adequate in vitamins.

S. Hirasawa (1923) reports that tryptophane is effective in treating various forms of experimental anemia in the rabbit. It had no effect upon normal animals. He found that histidine was ineffective when fed to such anemia rabbits. Minot and Murphy (1926) found that iron starvation anemia in infants may be remedied by food containing complete proteins and iron. These authors suggest that liver, leafy vegetables and fruits are effective in treating pernicious anemia because of the nature of their proteins. Koessler, Maurer and Laughlin (1926) claim that blood regeneration cannot take place in the absence of vitamin A, and that the rate of blood regeneration is a function of the amount of vitamin A present.

Whipple (1917–1928) and his associates, as a result of their numerous excellent studies, conclude that many tissues and fruits when fed to dogs stimulate hemoglobin regeneration and that some unknown constituent aids the utilization of proteins in the synthesis of hemoglobin.

Troensegaard and Schmidt (1924) have accumulated considerable experimental evidence that the protein molecule consists largely of heterocyclic rings in which the pyrrol structure predominates. He suggests that the precursors of the hematin nucleus of hemoglobin are to be found among these ring structures rather than in the amino acids which are obtained from these proteins by the usual methods of hydrolysis.

C. W. Saunders (1926) reported that young rats placed upon a synthetic diet containing 10 per cent gluten as the only protein constituent, became anemic, whereas a 10 per cent or 18 per cent casein diet produced a very high concentration of hemoglobin in the blood. When the casein of the diet had been extracted with 1 per cent acetic acid for eight weeks and subsequently dried on a hot plate a slight anemia was produced. This suggested that some of the precursors of hemoglobin had been destroyed or removed by the treatment and that possibly tryptophane might be the substance concerned. This point was studied in our first experiments.

In the first experiments described below, we attempted to produce a nutritional anemia in rats upon synthetic diets varying in their protein content. These attempts were unsuccessful. In later experiments blood regeneration in the rat was studied under conditions of severe experimental anemia and under various dietary conditions in which the protein and vitamin contents were varied.

EXPERIMENTAL PART. *Methods.* Blood volumes were estimated by the method described in our previous paper in THIS JOURNAL (1928). Hemoglobin was estimated by the Newcomer method upon blood samples obtained by cutting the rat's tail. Red corpuscle counts were made in the usual way.

Key to abbreviations used in the figures

B.V. per cent = cubic centimeters of blood per 100 grams body weight.
B.V. cc. = blood volume of the rat in cubic centimeters.
Hb. per cent = grams hemoglobin per 100 cc. of blood. = reading on Newcomer scale × 0.1692.
R.B.C. = red corpuscle count in millions per cubic millimeters of blood.
Wt. = body weight in grams.
Hb. value = grams of hemoglobin per 100 grams body weight. This is calculated from the blood volume, per cent of hemoglobin in the blood and the body weight.
Gm. Hb. withdrawn = the number of grams of hemoglobin removed at a single bleeding. This is calculated from the volume and hemoglobin concentration of the blood removed.

Hb. regeneration level = grams of hemoglobin produced daily by the rat over and above the maintenance factor. This is calculated from the amount of blood which is withdrawn to maintain the animal in its anemic state.

The quantitative values for the corresponding curves are keyed at the left margin of each chart.

A. Experiments with feeding gluten, casein and tryptophane. These experiments represent attempts to produce a nutritional anemia on a synthetic diet containing ten per cent wheat gluten, and to prevent such anemia by feeding tryptophane. The tryptophane fed in these experiments was optically active; $\alpha \frac{25}{D} = -32.0$. Casein was compared to gluten as a source of hemoglobin forming elements.

Fourteen young rats weighing 80 to 100 grams were fed upon the following diet: agar 5, dextrin 75, wheat gluten 10, Harris inorganic salt mixture 5, butter 5, Harris vitamin B extract of yeast 0.5. Six rats were fed on the same diet but in addition received 25 mgm. of tryptophane per rat daily. Five other rats received 60 mgm. of tryptophane per rat daily. Six rats received a diet similar to the 10 per cent gluten diet but casein was substituted for gluten. Six rats were fed upon an 18 per cent casein diet. Blood tests were made at one or two week intervals over a period of eleven to fourteen weeks. The results for typical rats of this series are given in figures 1 and 2. In the case of figure 1, the corresponding table of values is furnished to illustrate in detail the data obtained for rats 54 and 47.

An examination of figure 1 will show that the rats did not become anemic when maintained upon the 10 per cent gluten diet for seven weeks, but on the contrary showed an increase in hemoglobin. The two rats represented in figure 1 are typical examples of the fourteen rats on this diet. During the latter six weeks of the experiment the hemoglobin fell, but in no case was a true anemia encountered. This fall in hemoglobin per cent and hemoglobin value may have been due to a poor nutritional state of the animals due to a vitamin B deficiency. This was accidentally encountered because of the low potency of the sample of Harris vitamin B extract which we used. The question of vitamin B and anemia is taken up later in this article.

The curve for the rat receiving 60 mgm. of tryptophane daily is similar to the control in every respect. The tryptophane content of the wheat gluten was found to be 0.5 per cent. This means that the control rat 54 in figure 1 was eating approximately 5 mgm. of tryptophane per rat daily as compared to 65 mgm. in the case of rat 47 in figure 1. However, this difference produced no noticeable effect upon the hemoglobin of the rats. The fall in hemoglobin value after cessation of tryptophane feeding is not due to the removal of this substance from the diet since the controls showed

the same behavior. The results of this experiment indicate that tryptophane is no better utilized by the rat than is gluten for hemoglobin synthesis.

The rats in figure 2 are representative of those which were fed upon the 18 per cent casein diet. The blood changes in these rats are very similar to those of the controls in figure 1. However, the rats grow more rapidly upon this diet than upon the 10 per cent gluten diet. Although casein is

TABLE 1

Growing rats on a ten per cent gluten diet with and without added tryptophane

	FEBRUARY 13	FEBRUARY 15	FEBRUARY 21	MARCH 2	MARCH 14	MARCH 28	APRIL 4	APRIL 11	APRIL 24‡	MAY 6	MAY 10
Rat 54:											
Weight	98	102	104	107	101	92	86	81	81	92	83
Red blood cells	8.8		7.7	8.2	10.0	11.2		8.5	8.1		
Hemoglobin, per cent	10.7		12.5	12.9	13.9	15.9		13.9	11.7	10.7	
Blood volume, cc.		7.7					5.5				5.6
Blood volume, per cent		7.6					6.3				6.7
Hemoglobin value		0.81					1.0				0.72
	FEBRUARY 13	FEBRUARY 21	MARCH 2	MARCH 15	MARCH 28*	APRIL 5	APRIL 11	APRIL 24†	MAY 6‡	MAY 8	MAY 10
Rat 47:											
Weight	89	92	99	97	87	84	83	83	93		89
Red blood cells	7.5	7.1	7.6	9.0	9.1		7.4	8.9			
Hemoglobin, per cent	9.3	12.5	12.5	13.0	14.2		11.7	12.4	10.2		
Blood volume, cc.	5.8					5.2					5.2
Blood volume, per cent	6.5					6.2					5.8
Hemoglobin value	0.60					0.88					0.59

* Tryptophane dose increased to 60 mgm. March 25.

† Tryptophane feeding ceased April 13.

‡ Gave additional vitamin B starting April 27.

a more satisfactory protein than gluten from a nutritional standpoint, it is not superior to wheat gluten for promoting hemoglobin synthesis in the rat. Other experiments on a 10 per cent casein diet gave the same type of results.

The changes in blood volume in this type of experiment show the importance of controlling this factor, a precaution which is not often taken in rat experiments previously reported in the literature. It is not uncommon

to find that large changes in the hemoglobin concentration of the blood are balanced by inverse changes in the blood volume. Thus, if the blood volume factor is not controlled, we might conclude that anemia had de-

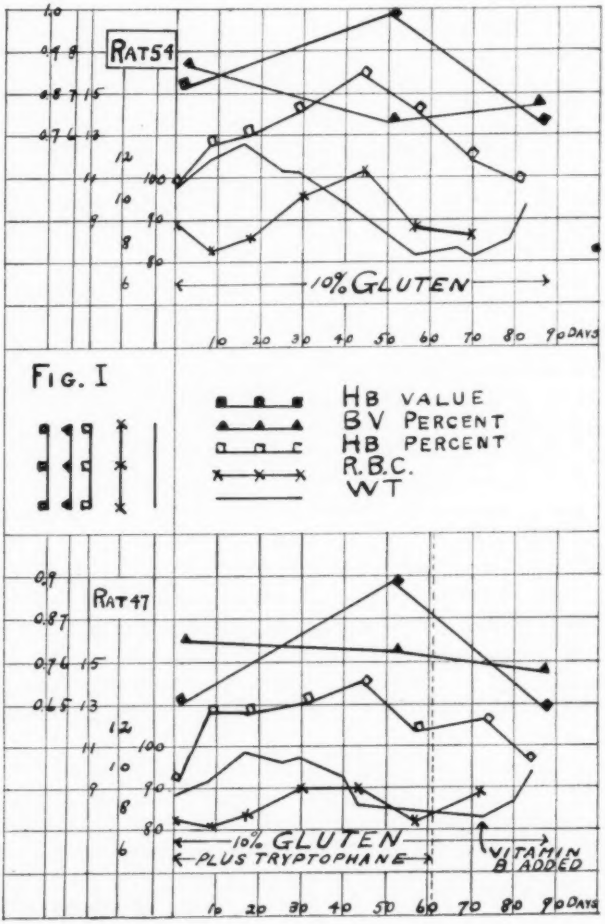


FIG. I

veloped or had been corrected when in reality there was no change in the number of grams of circulating hemoglobin per 100 grams body weight. However, even when the blood volume estimations are made, this type of experiment does not appear to be very satisfactory for anemia studies in

the rat. This led us to adopt a very different type of experiment in which severe anemia was produced in the rat by bleeding, and the rate of blood regeneration followed under various dietary conditions.

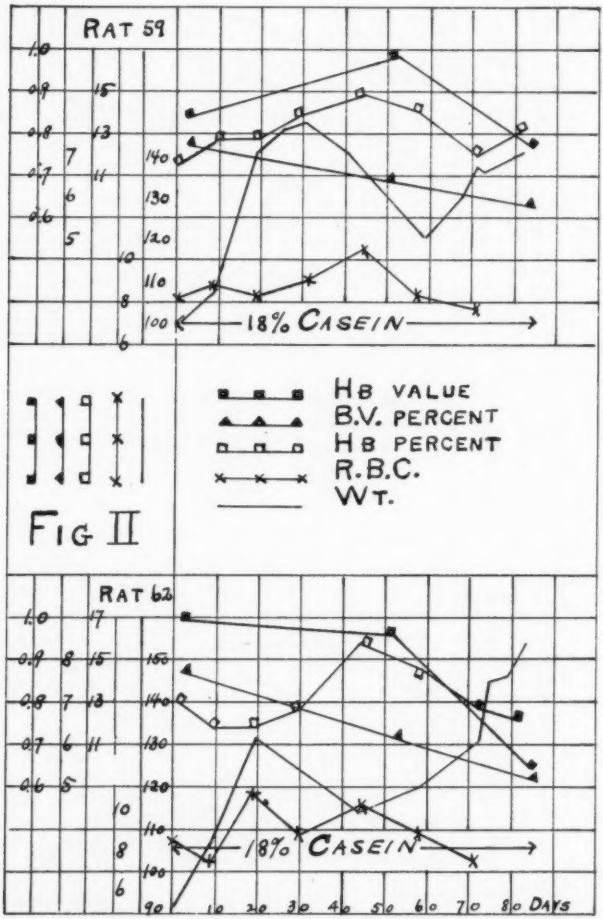


FIG II

B. Blood regeneration in the rat under conditions of severe anemia. The method used here on the rat is similar to that used by G. H. Whipple and his co-workers on the dog. The rat, however, has an advantage over the dog for such experiments in that it will eat and survive on a synthetic diet.

The hemoglobin level is reduced to approximately half its normal value and maintained at this level by repeated bleedings. Blood is drawn by cardiac puncture under ether anesthesia. Knowing the hemoglobin concentration and volume of the blood withdrawn, the amount of hemoglobin removed may be calculated in grams. If over any period of time the blood volume and hemoglobin concentration are the same at the beginning and end of this period, the number of grams of hemoglobin removed during this time is the amount of hemoglobin produced by the rat over and above the maintenance factor. Thus, we have a method for measuring the rate of hemoglobin production in the rat upon various diets and under conditions of maximum stress.

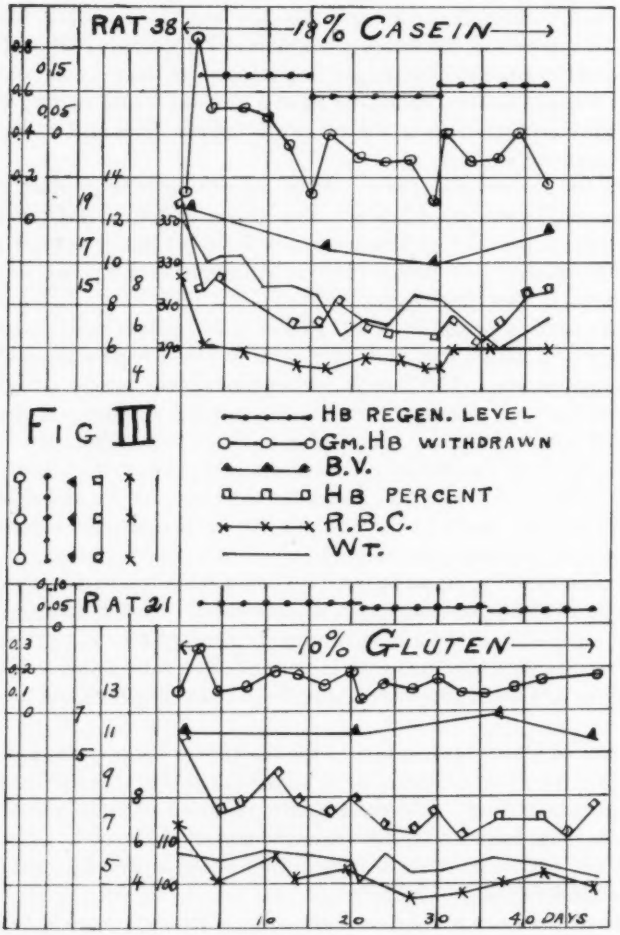
1. The ten per cent gluten diet. Since our attempts to produce a nutritional anemia in rats on a ten per cent gluten diet were unsuccessful, it seemed very doubtful that this protein is deficient in the organic precursors of hemoglobin. To obtain more information on this point, the severe anemia type of experiment was used.

Six rats varying in size from 107 grams to 365 grams were placed upon the 10 per cent gluten diet and their rates of blood regeneration followed as described above. The results for representative rat 21 are given in figure 3 and table 2. Each of these small rats when made anemic possesses 0.4 to 0.5 gram of blood hemoglobin. These rats regenerated hemoglobin under these conditions of severe anemia at the rate of 0.032 to 0.05 gram per day. This rate was maintained several weeks and was terminated only by the death of the animals due to hemorrhage after cardiac puncture. It was found necessary to bleed these rats to the extent of one-third of their blood volumes every third or fourth day in order to maintain the low hemoglobin concentration in the blood. This means that these rats are regenerating their blood completely every ten to thirteen days. Similar results were obtained with larger and older rats of this series. One of the older rats maintained the high rate of blood regeneration for twelve weeks.

This rate of blood regeneration in the rat upon a ten per cent gluten diet is comparable to that observed by Whipple in the dog on a diet containing large amounts of liver. The hemoglobin produced so rapidly under these conditions must have its precursors in the gluten of the diet. The results support our earlier conclusion that gluten is not a deficient protein for hemoglobin synthesis in the rat.

2. The eighteen per cent casein diet. Two adult rats were fed for eight weeks upon the following diet: agar 5, dextrin 66, casein 18, salts (Harris) 5, lard 4, cod liver oil 2, yeast extract. At the end of this time they were subjected to severe anemia and their rates of blood regeneration followed. The results for rat 38 are given in figure 3 and table 2 and are representative of the observations made on others.

These rats regenerated their blood every ten to fifteen days, which is no higher a rate than that observed in rats on a 10 per cent gluten diet. Casein is more efficient than gluten for promoting body growth, but



is no better than gluten for promoting red cell and hemoglobin production in the rat.

3. Feeding red corpuscle paste. Fresh, washed ox blood corpuscles were

used for these experiments. The paste contained 27 per cent hemoglobin. A preliminary experiment with the addition of corpuscle paste to gluten or casein diets on growing rats did not increase the hemoglobin value but stimulated growth.

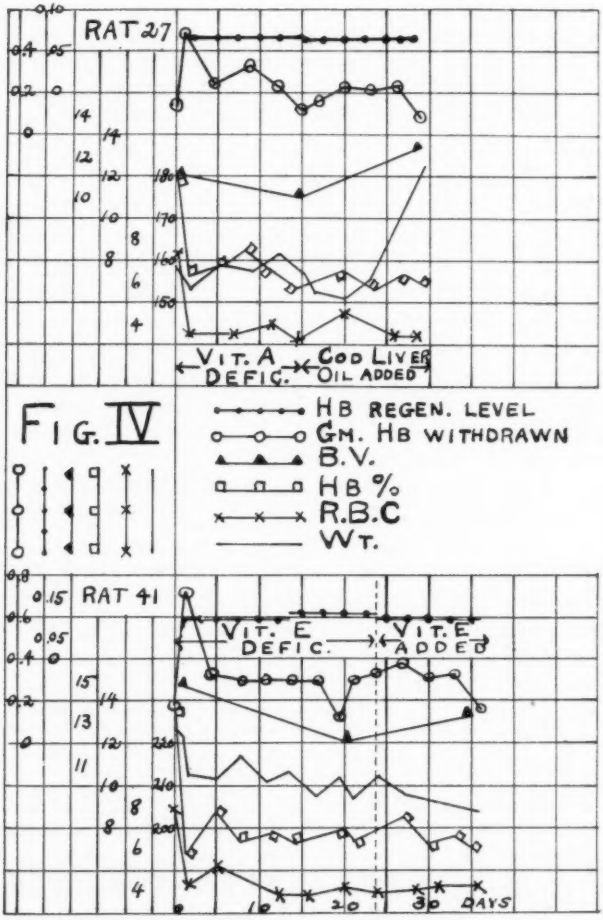


FIG. IV

This experiment was conducted to find out if the rat can utilize red blood corpuscles more easily in the diet for hemoglobin production under conditions of maximum stress. Two-week control periods were run upon two

TABLE 2

Blood regeneration on casein and gluten diets

RAT 38							RAT 21						
Date	Weight	Red blood cells	Hemoglobin	Blood volume	Blood drawn	Hemoglobin drawn	Date	Weight	Red blood cells	Hemoglobin	Blood volume	Blood drawn	Hemoglobin drawn
			per cent	*cc.*	*cc.*	*grams*				*per cent*	*cc.*	*cc.*	*grams*
June 22	290	7.5	15.9				June 8	107	6.7	11.3	6.1	0.7	0.08
August 16	352	8.9	13.2				June 10	107				2.8	
August 17	345			18.6	1.2	0.16	June 13	105	4.1	7.3		1.3	0.09
August 18	338				6.5	0.86	June 16	106		7.6		1.5	0.11
August 19	332	5.2	8.6				June 19	107	5.3	9.1		2.0	0.18
August 20	333		9.5		5.5	0.52	June 22	107	4.1	7.8		2.0	0.16
August 23	332	4.9	8.6		6.0	0.52	June 25	106		7.1		1.7	0.12
August 26	320		7.8		6.0	0.47	June 28	104	4.6	7.8		2.3	0.18
August 29	320	4.3	7.1		5.0	0.35	June 29	100			6.1	0.7	0.04
September 1	313	4.1	7.1	16.7	1.6	0.11	July 2	107		6.8		2.0	0.14
September 3	296		8.1		5.0	0.40	July 5	102	3.3	6.4		1.5	0.10
September 6	303	4.5	7.3		4.0	0.29	July 8	102		7.3		2.0	0.15
September 9	299		6.9		4.0	0.27	July 11	104	3.6	6.3		1.5	0.09
September 12	313	4.2	6.8		4.0	0.27	July 14	105		6.8	6.8	1.2	0.08
September 15	312	4.1	6.8	16.1	1.2	0.08	July 17	104		6.9		1.5	0.10
September 16	310	5.0	7.3		5.3	0.39	July 20	103	4.4	6.9		2.0	0.13
September 19	297		6.4		4.0	0.26	July 23	102		6.1			
September 22	290	5.0	6.8		4.0	0.27	July 26	101	3.8	7.5	5.8	2.0	0.15
September 25	297		8.0		5.0	0.40							
September 28	305	5.1	8.5	17.5	2.0	0.17							

rats at the end of which time the corpuscle feeding was begun. After seventeen days of corpuscle feeding, the control diet (10 per cent gluten) was resumed. The two rats of this experiment showed a progressive decline in the rate of blood regeneration which was not prevented by the addition of corpuscle paste to the extent of 15 per cent of the diet nor did the rate increase in the subsequent control period of fifteen days.

4. Vitamin A deficient diet. Eight rats, varying in weight from 65 to 170 grams, were placed on a vitamin A free diet consisting of casein 18, agar 5, dextrin 67, salts 5, lard 5, yeast extract. The casein was reprecipitated and extracted with hot alcohol and ether. After twelve weeks on this diet all of the rats had ceased to grow and two had died showing definite symptoms of vitamin A deficiency. None of the rats were anemic. The six remaining rats were subjected to severe anemia and their rates of blood regeneration followed. The results for one representative rat of this series are given in figure 4.

All of these rats when bled to the low hemoglobin level, regenerated their blood at the rate of once in every eight to twelve days, while maintained on a vitamin A deficient diet. Feeding 0.4 cc. of cod liver oil per rat daily did not alter the blood regeneration rate. No change in color index was observed. These results are opposed to those of Koessler, Maurer and Laughlin (1926) who report severe anemia in the rat on a vitamin A-free diet. These authors, however, base their conclusions in large part upon the histolotical blood picture, and failed to control the blood volume factor. Our results indicate that vitamin A is not directly related to the formation of either blood cells or of blood pigment in the rat.

5. Vitamin B deficient diet. Three rats weighing 200 to 250 grams were fed for eight weeks on a vitamin B deficient diet containing agar 5, casein 18, dextrin 66, salts 5, lard 4, cod liver oil 2. At the end of this time one was dead and the other two were losing weight and showing symptoms of vitamin B deficiency. The rats did not become anemic on this diet. They were subjected to severe anemia and their rates of blood regeneration followed.

While maintained on this diet, the rats regenerated their blood at the rate of once in every thirteen to fourteen days. They did not survive long enough to be used in testing the effect of adding vitamin B to the diet. Hence, the results of this experiment are inconclusive but indicate that blood regeneration can occur in the rat in the absence of vitamin B. The rate of blood regeneration appears to be slower on the vitamin B deficient diet than upon the vitamin A deficient diet, but the number of rats upon the vitamin B test is too small to warrant any conclusions upon this comparison.

6. Vitamin E deficient diet. The rats used in this experiment were born from parents on a vitamin E deficient diet and were raised upon this diet.

Mating tests with rats of known fertility showed that all of these rats were sterile. Four of these rats subjected to severe anemia as described earlier in this article, regenerated their blood at the rate of once every eight to twelve days. This rate is no lower than that of the rats in figure 3 receiving a casein diet not lacking in vitamin E. Furthermore, the feeding of 0.5 cc. of wheat germ oil per rat daily did not increase the rate of blood regeneration. These results indicate that vitamin E is not necessary for red cell or hemoglobin production in the rat. The results for one representative rat of this series are given in figure 4.

SUMMARY

Experiments are reported in which gluten and casein are compared as to their hemoglobin forming powers when fed to normal growing rats and to rats made severely anemic by bleeding. Rats on diets containing 10 per cent gluten or eighteen per cent casein can regenerate their blood completely every eight to fifteen days under the conditions of severe anemia described. Casein is no better utilized than is wheat gluten for hemoglobin production in the rat, and neither of these proteins is lacking in materials necessary for hemoglobin production. When the tryptophane intake of rats is varied between the limits of 5 mgm. and 65 mgm. per rat daily, no effect upon hemoglobin formation has been observed. We have not been able to obtain evidence indicating that tryptophane is a precursor of hemoglobin. Feeding red corpuscles does not appear to stimulate the rate of hemoglobin regeneration.

Rats fed for weeks on diets deficient in vitamins A, B or E do not become anemic. Furthermore, we have found that such rats when subjected to severe experimental anemia by bleeding, can regenerate their blood completely every eight to fifteen days. This rate is no lower than that observed upon similar diets complete in vitamins. We conclude that vitamins A and E and probably vitamin B, are not specifically essential for the hemoglobin forming process in the rat. The experimental evidence with reference to the value of vitamin B in experimental anemia in rats is not conclusive.

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